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13. ABSTRACT *(Maximum 200 Words)*

Changes in the expression of microfilament-associated proteins such as tropomyosins, are associated with the transformed phenotype. In this work we have tested the hypothesis that TM1 is putative biomarker and tumor suppressor of breast cancer. Using TM1-specific antibodies RNA probes, we are assessing the expression of TM1 in the primary breast tumor specimens.Restoration of TM1 expression in MCF-7 (MCF-7/T) cells resulted in slower growth rate. MCF-7/T cells remain sensitive to growth control by estrogen, and TM1 re-expression appears to strengthen the interaction of E-cadherin-catenin complex with microfilaments. More significantly, MCF-7/T cells failed to grow under anchorage-independent conditions. Thus, TM1 appears to be essential for normal growth and differentiation of mammary epithelium. Abolition of TM1 expression appears to be necessary for malignant transformation by multiple oncogenic modalities. Together, our findings demonstrate that TM1 is a class II tumor suppressor.

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Introduction

Normal cell morphology is maintained and regulated by actin microfilaments. The presence of disorganized or poorly structured microfilaments is a prominent feature of many transformed cells. Downregulation of microfilament-associated proteins, such as tropomyosins (TMs) is hypothesized to result in the formation of functionally aberrant microfilaments, thus contributing to the manifestation of the malignant cells. Previous studies from this laboratory have identified that: 1). Tropomyosin-1 (TM1) is a suppressor of the transformed phenotype, and; 2). TM1 is consistently abolished in a large number of breast carcinoma cells that are tested. The main objectives of the proposed research are to assess the expression of TM1 in the tissue specimens of breast cancer patients and to investigate whether TM1 functions as a suppressor of the malignant growth of breast cancer.

Annual Summary

Technical Objective 1: Analysis of TM1 expression in human malignant breast tumors and benign lesions, and normal breast tissues:

Normal mammary epithelial cells elaborate multiple isoforms of TMs (Bhattacharya et al., 1990) . The spontaneously transformed breast cancer cells were shown to exhibit severe deficiency in TM expression, with the expression of several TMs is either downregulated or completely abolished. For example, TM38 protein is expressed in MDA MB231 cells, but found to be absent in MCF-7 cells. Expression of TM1, however, was consistently lacking in all the breast carcinoma cell lines tested, indicating that TM1 suppression could be a common event during mammary carcinogenesis. Based on these data, we hypothesized that loss of TM1 is a critical biochemical change in the malignant transformation of breast epithelial cells and that TM1 could be used as a novel biomarker of breast cancer.

We have proposed to utilize a TM1-specific antibody that was generated in our lab for assessing TM1 expression in normal and malignant breast tissues. Since multiple TMs are present in epithelial cells, and the presence of stromal components which abundantly express TM1, we chose to analyze the tissues by immunohistochemistry. The smooth muscle cells of the blood vessels also express TM1. Currently available antisera recognize multiple isoforms of TMs and, thus, do not permit accurate analysis of TM1 expression. Therefore, we developed a TM1-specific antibody in this lab. A 20 amino acid sequence of TM1, which is distinctive of this isoform, was used as an immunogen and antiserum was raised against it. The resulting antibody, designated as TM-20, and tested in immunoblotting for its specificity Details of the antibody are presented in the manuscript (appendix).

We have tested the utility of these reagents in immunoblotting using cell lines with known TM expression profiles. For example, normal MCF-10A cells express all TM isoforms, including TM1 and TM38 which co-migrate on SDS-polyacrylamide gels. In MCF-7 breast carcinoma cells, neither of these proteins are expressed, while MDA MB231 cells express TM38, but not TM1. When TM1-specific antibodies were used in immunoblotting, only MCF10A cells showed reactivity, attesting to the specificity of the reagents.

We have used one such antibody, TM20, for immunohistochemical analysis. In the initial experiments, we found that TM20 showed positive staining with normal mammary epithelium, while the malignant ducts lacked/reduced reactivity. This antibody will be used for larger scale screening experiments at the Wake Forest University School of Medicine.

In the event that we encounter any problems with TM20 reagent in screening, we will use other antibodies available generated in this laboratory or develop RNA–based detection methods such as *in situ* hybridization or Laser Capture Microscopy-RT-PCR methods (Kuecker et al., 1999). For that purpose, we started the collection of fresh tissue immediately after the surgery to ensure good quality of RNA in the tissues. This objective will be accomplished before the end of the grant period as proposed. Another alternative would be to assess the methylation status of TM1 promoter, since the emerging work from this laboratory indicates that the TM1 gene is methylated in breast cancer cells.

Technical Objective 2: Effects of expression of TM1 in human breast carcinoma cell lines and in normal mammary epithelial cell lines.

Our previous studies with experimentally transformed murine fibroblasts have demonstrated that TM1 is a suppressor of the malignant transformation, and that TM1 is a class II tumor suppressor (Braverman et al., 1996; Prasad et al., 1999). To examine the role of TM1 in mammary carcinogenesis, and to determine whether TM1 can suppress the malignant growth of a spontaneously transformed human breast carcinoma cells, the following experiments were carried out. These data are communicated for publication, and the manuscript is attached in the appendix. Therefore, the results are briefly presented below.

MCF-7 cells, which lack TM1 protein, were transduced with a recombinant retrovirus expressing TM1. Individual cell lines expressing TM1 were isolated and tested for the effects of TM1 expression on the morphology and growth properties. Restoration of TM1 expression resulted in the formation of tighter colonies with a more branched, tubular appearance. The presence of TM1 containing microfilaments are readily detected. TM1 expression significantly decreased the growth rates, compared to parental MCF-7 cells. A more profound effect was observed on the anchorage-independent growth property, which is a hall mark of the neoplastic phenotype. TM1 expression completely abolished the anchorage independent growth of MCF-7 cells, indicating that TM1 suppressed the malignant growth properties. It should be noted that the revertant cells remain sensitive to the growth control by estrogen.

Initial investigations into the possible mechanisms of TM1 expression suggested that c-myc levels are lower in the revertant cells compared to MCF-7 cells. Furthermore, investigation of E-cadherin complexes revealed that no changes in the total expression of E-cadherin and catenins. However, it was found that E-cadherin and the catenins are more tightly associated with cytoskeleton of the revertants. Currently, we are investigating the role of TM1 in anchoring E-cadherin/catenin complex to the cytoskeleton.

With these experiments, a majority of the goals of this Objective are accomplished, and we completed the Objective 2, ahead of the schedule, indicated in the Statement of Work. Additional experiments in progress are directed at elucidating the mechanism of tumor suppression by TM1. These include studies on utilization of TM1, expression of TM1 in MCF10A cells, expression of TGFα and amphiregulin.

3. Induction of transformed phenotype by repression of TM1 of TM1 expression: In order to test whether the loss of TM1 expression could lead to the expression of malignant transformation of mammary epithelium, antisense suppression of TM1 is proposed. TM1 was subcloned in antisense direction in the retroviral vector pBNC and antisense packaging cells of PA317 are generated. The MCF10A cells have been transduced with the recombinant pBNC retrovirus. Transduced cells were selected for resistance to G418 and single cell clones were selected. These cell lines are now being tested for TM1 expression and their ability to grow under anchorage independent conditions. The work is progressing as per the Statement of Work.

At this point we are also considering more effective and novel strategies for antisense suppression. An alternative is the cre-lox mediated generation of TM1 knock out cell lines of normal mammary epithelial cells. For this purpose, we have isolated a BAC clone containing TM1 gene.

4. Structure-function relationship of TM1-mediated tumor suppression: Work on creating chimeras of TM1 (a tumor suppressor) and TM2 (not a tumor suppressor) has been initiated. We have completed the site directed mutagenesis to introduce a silent mutation to create a HindIII restriction site. This was accomplished by PCR and the resultant variants of TM1 and TM2, designated as 'TM1-h' and 'TM2-h' respectively, containing the HindIII site were sequenced. Switching of the carboxy (at AvaI) site and the central exons (HindIII-AvaI) is now in progress. We have encountered some problems in cloning the chimeras initially, which were identified during the verification of the chimeric constructs by sequencing. Therefore, a second attempt is being made and we expect to have the chimeric constructs at hand in about two more weeks.

Additionally, we are in the process of expressing TM2 in MCF- cells using recombinant pEE6 vector. This vector contains xanthine phospho ribosyl transferase as for selection against mycophenolic acid. This expression vector was found to be useful for expression of TM2 in fibroblasts. Furthermore, this vector would offer the flexibility if co-expression of TM1 and TM2 is desired for future experiments. In the pilot transient transfection experiments, TM2 expression was detected in MCF-7 cells. However, we are unable to select for the drug resistance in MCF-7 cells even after increasing the concentration of mycophenolic acid significantly (10μg/ml, used in fibroblasts, to 80μg/ml medium). Thus, it appears that MCF-7 cells appear to be refractory for selection with mycophenolic acid. Therefore, we are subcloning TM2 in to a different vector that utilizes G418 selection.

To expedite the selection of cells expressing TM2 and the chimeric TM constructs, we are employing a plasmid vector that coexpresses GFP via IRES sequences. This should enable us to fluorescently sort the populations of transfected cells and obtain homogenous populations. This modification should obviate the need for single cell cloning of each of MCF-7 cells expressing the chimeric TMs. Transfected cells will be sorted for GFP expression and expanded in presence of G418. The mass cultures will be tested for the expression of chimeric TMs (and TM2, as appropriate). Growth properties of these cultures will be studied in monolayer as well as in soft agars.

We propose to transfect the MCF-7 cells with the first chimeric TMs to identify the domains of TM1 that are responsible for tumor suppression. This will be a variation from the Statement of Work, where we proposed to carry out baculoviral expression and purification after the constructs are cloned. This modification will allow us to immediately assess the effects of the chimeric TMs on the growth phenotype of MCF-7 cells.

At this point, in parallel, we have chosen to test the use of epitope-tagged TMs in a different cell system. Several investigators have used epitope-tagged constructs to monitor the transfected genes. Epitope tagging of TM1 is expected to greatly facilitate the analysis of transfected TMs. To confirm that epitope tagging does not impair the tumor suppressive function of TM1, we chose to test the constructs in DT cell (ras-transformed

NIH3T3 cells) system. Our previous published studies showed that DT cells are suitable models to test the ability of TM1 to reorganize the cytoskeleton and tumor suppression. A HA epitope was engineered to the amino terminus of TM1. The ATG initiation codon of TM1 was replaced with an oligonucleotide sequence encoding HA epitope. The recombinant TM1 was subcloned into a eukaryotic expression vector, and transfected into DT cells. Detailed characterization of the transfected cells is in progress. Depending on those results, we will either continue to work with the constructs that are being generated, or employ the epitope-tagged constructs for easy monitoring in the transfection experiments.

Key Research accomplishments

- Several TM1-specific antisera have been developed and their specificity is tested.
- Screening of breast tissues for TM1 expression is in progress.
- In addition to originally proposed immunohistochemical screening, we are now employing *in situ* hybridization and Laser Capture microscopy-RT PCR strategy. Conditions for in situ hybridization are standardized, and sample collection is in progress. We are now determining the conditions for quantifying the RT-PCR.
- Restoration of TM1 expression reverts MCF-7 cells.
- The revertant MCF-7 cells display improved cell-cell adhesion complexes, with e-cadherin and β-catenin associated more tightly to the cell-cell junctions.
- A related research finding is that restoration of TM1 expression in MCF-7 results in profound decrease in the MAP kinase activity. This and other signal transduction studies in progress are not proposed in the grant application. However, these experiments should provide important mechanistic clues how TM1 reverts breast cancer cells.

Reportable Outcomes

Manuscripts and Abstracts

1. Manuscripts:
   1.1. Abolition of Tropomyosin-1 gene expression in breast cancer cells by methylation and histone deacetylation. Shantaram Bharadwaj and G. L. Prasad (manuscript in preparation).
   1.2. Suppression of transformed phenotype of breast cancer by tropomyosin-1. Kalyankar Mahadev, Gira Raval, Mark Willingham, Barbara Vonderhaar, David Salomon and G. L. Prasad (manuscript communicated).
   1.3. Vanya Shah, Shantaram Bharadwaj, Kozo Kaibuchi and G. L. Prasad (2001) Cytoskeletal organization in Tropomyosin-mediated reversion of ras-transformation: Evidence for Rho kinase pathway. Oncogene 20: 2112-2121.
   1.4. Edward A. Levine, and G. L. Prasad. Oncology. In The Physiologic basis of Surgery (Ed. O'Leary). 2001.
2. One abstract was presented:
   2.1. Tropomyosin-1 is a suppressor of the malignant phenotype of breast cancer cells. (2001) G. L. Prasad S. Bharadwaj, G. N. Raval, and B. K. Vonderhaar. Gordon conference on Mammary Gland Biology.

The U.S. Army Medical Research and Materiel Command under DAMD-17-98-1-8162 supported this work.

Conclusions

In summary, the major accomplishment of our work for this year is to demonstrate that TM1 indeed is a tumor suppressor of breast carcinoma cells. We showed that restoration of TM1 expression in MCF-7 model cell system, abolishes anchorage-independent growth, significantly decreases the growth rates, while not altering the estrogen growth controls. We also found that TM1 might mediate these effects through E-cadherin-βcatenin pathway (Polakis, 2000; Yap et al., 1997).

These findings could have important implications in understanding biology of breast cancer and possibly exploring novel therapies. First of all, our studies indicate that the assembly of cell-cell adhesion junctions can be modulated by changes in microfilament associated proteins. This suggests that microfilament proteins through their ability to regulate the assembly of cadherin-catenin junctions could, in fact, alter the gene expression via TCF-LEF/-βcatenin pathway (Barker et al., 2000). This possibility sheds light on the TM1-mediated suppression of the revertant phenotype, which is being investigated.

A second implication is to test whether TM1 can be used for gene therapy of breast cancer. Since TM1 appears to be a transformation-specific suppressor, it is an attractive therapeutic target. We are planning to explore this possibility by employing adenoviral vectors for the gene delivery using Adeasy adenoviral vectors (He et al., 1998). We have generated the recombinant adenoviral vector designed to express TM1. Additionally, we will use the adenoviral expression system as an inducible vector to investigate the early events in tumor suppression by TM1.

References

Barker, N., Morin, P. J., and Clevers, H. (2000). The Yin-Yang of TCF/beta-catenin signaling. Advances in Cancer Research *77*, 1-24.

Bhattacharya, B., Prasad, G. L., Valverius, E. M., Salomon, D. S., and Cooper, H. L. (1990). Tropomyosins of human mammary epithelial cells: consistent defects of expression in mammary carcinoma cell lines. Cancer Research *50*, 2105-12.

Braverman, R. H., Cooper, H. L., Lee, H. S., and Prasad, G. L. (1996). Anti-oncogenic effects of tropomyosin: isoform specificity and importance of protein coding sequences. Oncogene *13*, 537-45.

He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. Proceedings of the National Academy of Sciences of the United States of America *95*, 2509-14.

Kuecker, S. J., Jin, L., Kulig, E., Oudaogo, G. L., Roche, P. C., and Llyod, R. V. (1999). Analysis of PRL, PRL-R, TGFb1, and TGFb-RII gene expression after Laser Capture Microdissection. Applied immunohitochemistry & Molecular Morphology *7*, 193-200.

Polakis, P. (2000). Wnt signaling and cancer. Genes & Development *14*, 1837-51.

Prasad, G. L., Masuelli, L., Raj, M. H., and Harindranath, N. (1999). Suppression of src-induced transformed phenotype by expression of tropomyosin-1. Oncogene *18*, 2027-31.

Yap, A. S., Brieher, W. M., and Gumbiner, B. M. (1997). Molecular and functional analysis of cadherin-based adherens junctions. Annual Review of Cell & Developmental Biology *13*, 119-46.

Suppression of the transformed phenotype of breast cancer by tropomyosin-1.

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Key words: Tropomyosin, cytoskeleton, E-cadherin, β-catenin, breast cancer

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Abstract

Changes in the expression of microfilament-associated proteins such as tropomyosins (TMs), are associated with the transformed phenotype. Our previous work demonstrated that: 1. the loss of expression of tropomyosin-1 (TM1) is a common biochemical event found in many malignantly transformed cells, and; 2. TM1 is a suppressor of the malignant phenotype induced by retroviral oncogenes. We also showed that TM1 expression is consistently abolished in the human breast carcinoma cell lines. In this work we have tested the hypothesis that TM1 is a tumor suppressor of breast cancer.

MCF-7 cells, which have lost the expression of TM1, were utilized as a model of human breast cancer. Restoration of TM1 expression in MCF-7 cells (MCF-7/T) resulted in slower growth rate, but remain sensitive to growth control by estrogen. More significantly, MCF-7/T cells failed to grow under anchorage-independent conditions. TM1 re-expression alters the interaction of E-cadherin-catenin complex with the cytoskeleton, indicating that TM1-induced microfilaments could play a significant role in suppression of the malignant phenotype. Thus, TM1 appears to be essential for normal growth and differentiation of mammary epithelium.

Introduction:

During the neoplastic transformation, cells accumulate several different mutations and undergo extensive changes in gene expression. While the etiology of the vast majority of tumors is unknown, it is generally accepted that multiple genetic events contribute to the neoplastic transformation of cells (Kinzler & Vogelstein, 1997). Some of the changes include loss of tumor suppressor genes, activation of cellular proto-oncogenes, and inactivation/deregulation of the function or expression of key intracellular proteins. The mortality due to cancer is primarily a consequence of the ability of the neoplastic cells to invade and metastasize in tissues where the primary cells do not normally grow. Tumor metastasis involves multiple steps including the loss of normal growth controls, the derangement of cytoskeletal organization, and the capacity to become motile and invasive (Bissell *et al.*, 1999; Hirohashi, 1998; Sommers, 1996). It has been postulated that deregulation of growth factor (serum)-controlled and integrin-regulated adhesion pathways together contribute to the complete transformation involving accelerated proliferation and anchorage independent growth (Schwartz *et al.*, 1997). In addition, tumor cells also manifest altered cell-cell adhesion and abnormal microfilaments, which facilitate invasion. Microfilaments are linked to both integrin and cadherin-catenin complexes which regulate cell-matrix and cell-cell adhesion respectively.

Our work on the role of cytoskeletal proteins in cell transformation has demonstrated that derangements in tropomyosin (TM) expression are a common biochemical change in many breast carcinoma cells (Bhattacharya *et al.*, 1990). This observation extends the earlier findings that the loss of tropomyosin expression is commonly found in many experimentally transformed fibroblasts (Cooper *et al.*, 1987; Cooper *et al.*, 1985; Lin *et al.*, 1997; Matsumura *et al.*, 1983). TMs are a family of closely related actin-binding proteins (Lin *et al.*, 1997; Pittenger *et al.*,

1994). Multiple isoforms of TMs are expressed from four genes via alternate splicing in a highly tissue specific manner (Lin *et al.*, 1997; Pawlak & Helfman, 2001; Pittenger *et al.*, 1994). TMs bind to actin filaments and stabilize them against the action of gel severing proteins such as gelsolin (Ishikawa *et al.*, 1989a; Ishikawa *et al.*, 1989b). The 284 amino acid high M_r TMs generally display higher affinity to actin than the low M_r isoforms (248 aa) (Pittenger *et al.*, 1994). Other important cytoskeletal proteins such as caldesmon, fascin and tropomodulin also modulate TM interactions with actin (Ishikawa *et al.*, 1989b; Yamashiro *et al.*, 1998).

Although it is known that suppression of high M_r TMs is a prominent feature of many experimentally transformed murine cell lines, the relevance of TMs in human cancers is largely unknown. To that end, we have investigated the role of TMs in mammary carcinogenesis. In normal human mammary epithelial cells, 7 different isoforms of TMs are expressed (Bhattacharya *et al.*, 1990). Among these, TM1, TM2, TM3 and an epithelial cell type specific species TM38, may be categorized as high M_r TMs. Isoforms TM32a, TM32b and another epithelial specific protein, TM32, are known to be low M_r TMs. In spontaneously transformed human breast carcinoma cell lines, loss of expression of multiple isoforms of TMs has been reported. More significantly, expression of TM1 is completely abolished in all the transformed cell lines, suggesting that suppression of TM1 could be a pivotal event leading to the acquisition of the neoplastic phenotype by mammary epithelial cells.

Furthermore, it was demonstrated, using oncogene-transformed murine fibroblasts, that restoration of TM1 expression is adequate to revert the malignant phenotype induced by functionally diverse oncogenes such as *ras* and *src* (Braverman *et al.*, 1996; Prasad *et al.*, 1993; Prasad *et al.*, 1999). These data suggest that TM1 could be a general suppressor of cellular transformation, regardless of the initial transforming events. TM1, therefore, may be classified as

a class II tumor suppressor (Schafer, 1994). Other class II tumor suppressors include Rsu-I (Cutler *et al.*, 1992). Inactivation of class II tumor suppressors may be necessary for acquiring the malignant phenotype, and this is likely to be a result of the inactivation of the class I tumor suppressors such as Rb or p53. Additionally, activation of cellular oncogenes could also decrease the expression or inactivate class II tumor suppressors (Sers *et al.*, 1997).

The experimentally transformed fibroblasts employed in the above studies to define a causal relationship of TMs to cell transformation are generally generated by a single well-defined transforming oncogene. Most human cancers, on the other hand, originate in epithelial cells as a result of multiple genetic defects. Furthermore, mechanisms that lead to transformation of epithelial cells could be different and more complex. For example, while both *raf* and *ras* transform fibroblasts, epithelial cells can only be transformed by *ras* (Oldham *et al.*, 1996). Another complexity with epithelial cells is that, at least two more TM isoforms are expressed in epithelial cells compared to fibroblasts, which could potentially compensate for loss of the other TMs. Therefore, it remains to be established whether TM1 can function as a suppressor of the malignant phenotype of spontaneously transformed, human-derived carcinoma cells. To further investigate the role of TM1 in human cancers, we determined whether restoration of TM1 expression in the MCF-7 human breast cancer cell line has an effect on the growth and transformation of these cells.

Materials and Methods

Normal mammary epithelial MCF10A cells were obtained from Dr. Jose Russo, Fox Chase Cancer Center, Philadelphia (Prasad *et al.*, 1992) and MCF-7 cells were purchased from ATCC. The cDNA encoding TM1 protein has been previously described (Prasad *et al.*, 1991).

Anti-TM polyclonal antiserum which recognizes multiple TMs, including TM1, was described previously (Bhattacharya *et al.*, 1990). DT/TM1 and DTD/TM1-TM2 cells were fibroblast cell lines and previously described (Shah *et al.*, 2001; Shah *et al.*, 1998).

For retroviral gene transfer of TM1 into MCF-7 cells, a pBNC recombinant virus was used as described (Prasad *et al.*, 1994; Prasad *et al.*, 1993), except that an amphitrophic packaging cell line PA317 was used to generate infectious virus. TMe1 cDNA was subcloned into a pBNC retroviral vector in which a CMV intron/enhancer drives the expression of the gene of interest and the selection is accomplished with G418. Transduced MCF-7 cells were cloned by a limiting dilution method and the cell lines (MCF-7/T) were tested for TM1 expression. For control purposes, MCF-7 cells were transduced with the empty vector and the resultant cell lines were designated as MCF-7/V cells.

Protein analysis: Two dimensional gel electrophoresis using cell lysates prepared from metabolically labeled cells was performed essentially as described previously (Bhattacharya *et al.*, 1990; Cooper *et al.*, 1985; Prasad *et al.*, 1994). Western blotting was performed with TM antiserum or commercially available antibodies. Expression of α-tubulin was routinely measured with a specific antibody (Sigma chemical Co) as a load control. Antibodies against E-cadherin and catenin were purchased from Transduction Laboratories. For routine analysis, cells were extracted with a 50mM Tris buffer pH 7.4, containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.15M NaCl, 1mM EDTA, 1mM sodium fluoride, 1mM sodium orthovanadate and a cocktail of protease inhibitors (1mM PMSF and 1μg/ml each of leupeptin, pepstatin and aprotinin). After a brief centrifugation to remove nuclei and cell debris, the supernatant was used for immunoblotting or immunoprecipitations. These lysates are termed cytoplasmic preparations. Alternatively, cells were also extracted with RIPA buffer (RIPA lysates) (Kinch *et al.*, 1995). To

extract both nuclear and cytoplasmic proteins, cells were solubilized with 1% SDS and used for immunoblotting (whole cell lysates).

A 20 amino acid sequence (187-206; SRARQLEEELRTMDQALKSL) (19) that is distinctive for TM1 was used to generate a TM1-specific antibody in rabbits. The antiserum was purified on a peptide immunoaffinity column and used for immunoblotting. It detects only TM1 on immunoblots. The polyclonal anitserum which recognizes all the TM isoforms was previously described and used for immunoblotting, immunocytochemistry and immunoprecipitations (Prasad *et al.*, 1999; Shah *et al.*, 1998)

Northern analysis: Total cellular RNA was analyzed as previously described (Braverman *et al.*, 1996). The membranes were probed with a full length TM1 cDNA (Prasad *et al.*, 1993; Prasad *et al.*, 1991). The blots were washed at 65^0C in a buffer containing 0.1x SSC and 1% SDS. Under these conditions, only TM1 is detected without any cross hybridization to other TMs. Blots were then stripped and reprobed with β-actin for load controls.

Monolayer Growth: Growth of the cells were measured in monolayer. Briefly, 2×10^5 cells were plated in normal (10%) serum. At regular intervals, cells were harvested and counted using a hemocytometer. Cell culture conditions were previously described for MCF-7 cells (Prasad *et al.*, 1992), and the medium for transduced cells contained 200μg/ml of G418. Experiments involving estrogen deprivation and supplementation were performed in a phenol red-free basal medium. Two replicate plates were initiated for each cell type and growth median. To test the effects of estrogen on the growth of the cells, 72h after plating, normal medium was replaced with a medium containing charcoal stripped FBS alone (minus estrogen), or with a supplementation of 100nM 17-bestradiol (plus estrogen). Cell count measurements were subsequently taken on each plate every 24 hours (total five measurements over 120 hours).

Mixed model analysis of variance (ANOVA) was performed, adjusting for estrogen exposure, to test for equality of growth after 120 hours between the different cell types. Counts on the same plates were treated as a correlated measurements in the statistical analyses. Statistical analysis was performed using the SAS v8.1 software package (SAS, Cary, NC).

Anchorage independent growth: Five thousand cells were plated in soft agar as described (Prasad *et al.*, 1993). Cells were fed once in 48h with 0.1ml of medium. Six replicate plates were cultured for each cell type. Two weeks later, cells were stained with 0.5% nitroblue tetrazolium (Sigma). Colonies ≥50 microns were counted. Equality of the mean number of counted colonies after two weeks of incubation between the different cell types was tested using t tests.

Immunofluorescence: Cells were cultured in Nunc chamber slides, fixed with 3.7% paraformaldehyde (Shah *et al.*, 1998) and permeabilized with 1% Triton X-100 in phosphate buffered saline (PBS). For E-cadherin staining, samples were briefly extracted with 1% Triton X-100 and then fixed in paraformaldehyde, and reacted with primary antibody. The samples were blocked in 1% BSA (Jackson Immunolabs) in PBS and incubated with primary antibodies overnight at 4^0C. The TM antiserum was used at 1:500, and other commercial antibodies were diluted as per the manufacturer's guidelines. The samples were also stained with Texas-Red conjugated phalloidin (Molecular Probes) for some experiments. The samples were mounted using Prolong Antifade kit (Molecular Probes). Confocal microscopy was performed with a Zeiss confocal microscope with a 60X water objective. The images were optically sectioned and the composite images are projected.

For determination of the intensity of staining, the samples were viewed with a Zeiss Axioplan 2 microscope using either a 40x or 63x oil objectives. The images were captured using a Dage MTI camera (model 300) and IFG 310 controller. The samples were photographed using

different gate settings, which allows accumulation different of numbers of frames. The gate setting is inversely linked to the brightness of staining. For example, if a gate setting of 4 is used to photograph, 4 different individual frames will be taken and integrated in a final image. However, if a gate setting of 8 is required, it suggests that the image is about half as intensely stained as the first one. In these experiments, both the gain and black level, which affect the image quality, were kept at identical settings. The images were transferred to Adobe Photoshop and processed identically to make a composite image. Further quantification of the signal, such as area and intensity measurements using Photoshop were not done, as this is a semi quantitative method. Multiple areas of the sample were photographed using gate settings 4, 8, 16 and 32, depending on the intensity of the signal.

Results:

Restoration of TM1 expression in MCF-7 cells: Normal mammary and other epithelial cell types express 7 different TMs, two more TMs than found in fibroblasts, that are readily detected in 2-D gels (Bhattacharya *et al.*, 1990; Prasad *et al.*, 1991). TM expression in nontransformed, immortalized human mammary epithelial MCF10A cell line is shown in Figure 1A. One of these additional TMs, TM38, co-migrates with TM1 in one dimensional gels (Figure 1B). The other TM isoform, TM32, also is not resolved from the two low M_r TMs in routine SDS PAGE, but requires 2-D analyses. Both of these TM isoforms are not well characterized. In malignant breast epithelial cells, TM1 expression was consistently absent, but the expression of other TMs including that of TM38 varies. For example, in MDA MB231 and MDAMB453 cells TM38 expression is detectable by immunoblotting with polyclonal anti-TM antisera, and by 2-D gels (Figure 1B, top panel; (Bhattacharya *et al.*, 1990). However, in MCF-7 cells both TM1 and

TM38 are lacking, and, therefore, no signal is detected. The fibroblast cell lines, DT/TM1 and DT/TM1-TM2, which express either TM1 alone, or TM1 and TM2 together, respectively are used as positive controls for TM1 expression. These cells lack the expression of TM38, and hence, the signal in the fibroblast-derived cell lines indicates the expression of TM1 (Bhattacharya *et al.*, 1990; Prasad *et al.*, 1993; Prasad *et al.*, 1991; Shah *et al.*, 1998).

To facilitate the analysis of TM1 expression, we generated a specific anti-peptide antiserum, as described in Materials and Methods. Data from immunoblotting with TM1-specific antibody are shown in Figure 1B (middle panel). The two fibroblast cell lines DT/TM1 and DT/TM1-TM2, and the MCF10A cells contain readily detectable TM1. In agreement with two dimensional gel analyses , expression of TM1 was lacking in MCF-7, MDA MB231 and MDA MB453 breast cancer cell lines cell lines (Bhattacharya *et al.*, 1990). In MDA MB231 and MDA MB453 cells which express TM38, no signal was detected by TM1-specific antibody, indicating the lack of TM1. MCF-7 cells that were transduced to re-express TM1, designated MCF-7/T, however, was positive for TM1 expression (discussed below).

In MCF-10A human mammary epithelial cells, TM1 is expressed from a 1.1kB mRNA. In MCF-7 human breast carcinoma cells, expression of both TM1 protein and its cognate mRNA is totally abolished, as is the case with several other breast carcinoma cells (Figure 2A; also Figure 1B) (Bhattacharya *et al.*, 1990). Transduction of MCF-7 cells with a recombinant retroviral vector results in the expression a 2.0kb mRNA from which TM1 is transcribed, as found in MCF-7/T cell lines; TM1 mRNA is of 1.1kB in size, while the additional sequences originate from the vector (Prasad *et al.*, 1993). We have analyzed three independent MCF-7/T cell lines, and two MCF-7/V cell lines along with the parental MCF-7 cells. In MCF-7/V cell

lines (vector controls) and the parental MCF-7 cells, the transduced 2.0kb mRNA is absent, as expected.

Immunoblotting of cytoplasmic lysates with a polyclonal anti-TM antibody revealed that TM1 is readily detectable in MCF10A cells. However, no corresponding signal was present in MCF-7 or MCF-7/V cells. In MCF-7/T cell lines TM expression is restored (Figure 1B, middle panel; data not shown for other cell lines). Since high M_r TMs do not resolve well in one dimensional gels and the individual TMs significantly vary in their avidity to antibody, we examined total expression of TMs in these cell lines. To that end, cellular extracts prepared from metabolically labeled cells were analyzed by two-dimensional gels (Figure 2B). In MCF-7 cells, expression of TM1, TM38 and TM2 is abolished, and therefore, none of these three proteins are detected (Bhattacharya *et al.*, 1990). Among the muscle-type high M_r TMs that are present in epithelial cells, only TM3 is present in MCF-7 and MCF-7/V cells. Transduction of MCF-7 cells with TM1 cDNA, as expected, results in a specific enhancement of TM1 protein in MCF-7/T cells. Furthermore, transduced TM1 is also found in the cytoskeletal fraction of MCF-7/T cells (data not shown).

Morphology of MCF-7/T cells: Restoration of TM1 expression in MCF-7 cells resulted in significant morphological changes. MCF-7 cells and the vector control cells shows that they grow as rather loosely adhering clusters. MCF-7/T cells in general grow in tighter clusters and form distinctive tubular structures (Figure 3A) (Kamarainen *et al.*, 1997).

Immunocytochemical staining with anti-TM antibody of parental MCF-7 cells showed weak staining with the TM antiserum although MCF-7 cells express at least one high Mr TM isoform and low M_r TMs. In these cells, TM staining is faint and diffuse throughout the cell, with no detectable association with actin filaments (Figure 3B; panels A-C). In MCF-7/T cells, TM

staining was intense, and TM containing microfilaments were evident. In addition, TM staining colocalized with that of actin, indicating that transduced TM1 reorganizes microfilaments (Figure 3B; panels D-F). Although TM staining was found through the cell body, it was brightest around the nucleus. TM staining was generally absent along the periphery of the cell.

Growth Properties: The effect of TM1 expression on the growth properties of MCF-7 cells was assessed in monolayer cultures. MCF-7, a vector control cell line (V1), and three cell lines expressing TM1 (T1, T2 and T3) were used to measure the growth. Under normal serum conditions, the unmodified MCF-7 and those transduced with empty vector grew rapidly at similar rates (Figure 4 A). However, all the three individual cell lines expressing TM1 grew comparably, but demonstrated strikingly slower growth after 120 hours than the parental MCF-7 or MCF-7/V cell lines ($p < 0.0001$ for all pairwise comparisons). Thus, restoration of TM1 expression decreases the proliferation of breast carcinoma cells.

Estrogen regulates the growth and differentiation status of MCF-7 cells. Since TM1 expression decreases the growth of these cells, we tested whether MCF-7/T cells remain sensitive to growth controls by estrogen (Figure 4B and Figure 4C). When cells were cultured in the absence of estrogen using charcoal stripped fetal bovine serum, growth of all the cell lines, including those expressing TM1, was inhibited by about 50%. Even under these conditions, MCF-7 and MCF-7/V1 cells (Figure 4B) maintained higher growth than MCF-7/T cells, as shown in Figure 4C ($p < 0.0001$ for all pairwise comparisons). Supplementation with 100nM bestradiol in charcoal-treated serum containing medium resulted in increased growth. In the presence of estrogen, MCF-7 and MCF-7/V1 cells demonstrated profoundly enhanced growth compared to MCF-7/T cells ($p < 0.0001$ for all pairwise comparisons). Addition of 5-hydroxytamoxifen, inhibited the stimulatory effect of estrogen (data not shown). These data

show that restoration of TM1 expression decreases the growth of MCF-7 cells, without altering the sensitivity to estrogen.

Anchorage independent growth: The proliferation of normal cells is tightly regulated by growth signals of integrin-extracellular matrix interactions, which is often deregulated in tumor cells. The ability of tumor cells to proliferate independent of adhesion closely correlates with tumorigenic potential, which is often assayed by anchorage-independent growth in soft agar. The data presented in Figure 4 show that re-expression of TM1 decreases the growth of MCF-7 cells. Therefore, we examined whether TM1 inhibits anchorage-independent growth of MCF-7 cells. MCF-7 and V1 and V2 cells grew rapidly and formed a large number of colonies within 2 weeks (Figure 5A and 5B). In contrast, the three clones of MCF-7 cells expressing TM1 did not grow in parallel cultures. The MCF-7/T cell lines showed a significant decrease in growth in the number of colonies ($p < 0.0001$ for all comparisons between MCF-7, V1, and V2 cells and MCF-7/T cells). These data demonstrate that re-expression of TM1 abolishes the malignant potential of breast carcinoma cells and support our earlier studies on the anti-transformation effects of TM1.

Cell-cell adhesion molecules in the revertant breast cancer cells: Expression of E-cadherin is down regulated in several human malignancies, including breast cancer. Decrease in E-cadherin expression weakens cell-cell interactions, and contributes to the metastatic phenotype. Additionally, E-cadherin complexes with α, β and γ-catenins, and the entire complex is anchored to microfilaments. The cadherin-catenin complex, is implicated in regulating tissue integrity, polarity and differentiation. Stable association of cadherin-catenin complex to microfilaments is considered as a requirement for normal functioning of cadherin complexes (Gumbiner, 2000; Yap *et al.*, 1997). Furthermore, free, soluble β-catenin, which is not associated with cadherins, is a key player in the wnt signaling pathways (Polakis, 1999; Polakis *et al.*, 1999). Activation of

wnt signaling pathways, results in the transportation of β-catenin into the nucleus and interaction with TCF/LEF transcription factor thereby upregulating gene expression. Since, cells expressing TM1 form tighter clusters and display a tubular morphology indicating enhanced differentiation, we investigated whether TM1-induced reversion of breast carcinoma cells involves changes in the expression of E-cadherin or the catenins.

Cytoplasmic lysates were prepared from actively growing cells and were immunoblotted with antibodies against E-cadherin and α-, β-, and γ-catenins. The data of Figure 6A indicate that MCF-7 and the two vector control cell lines express similar levels of all the four proteins tested. The three cell lines of revertant MCF-7/T, however, showed significantly and consistently lower levels of E-cadherin and the catenins (Figure 6A). Since TM1 expression is associated with the re-emergence of microfilaments, we investigated a possibility that the cadherin complex may be more tightly associated with the cytoskeleton in the revertants, thus, forming stronger cell-cell junctions which may make them less soluble. To examine the total expression of these proteins, cells were extracted under more vigorous conditions, using RIPA buffer to lyse the cells, and tested for the presence of the components of cadherin-catenin complex. Under these conditions, no detectable differences in the expression of E-cadherin or the catenins were found between the transformed (MCF-7 and V) and the revertant TM1-expressing MCF-7/T clones (Figure 6B): similar results were obtained when cells were solubilized with 1% SDS and lysates were analyzed (data not shown). These results indicate that in MCF-7/T cell lines, E-cadherin and the catenins associate more tightly to the cytoskeleton, presumably contributing to the stability of cell-cell adherens junctions.

To investigate whether the localization of E-cadherin and the catenins is altered in the revertants, immunocytochemistry was performed. In MCF-7 cells, β-catenin (Figure 7A; panels

A) and E-cadherin (Figure 7A; panel B) were detectable at the cell-cell junctions. In MCF-7 cells, E-cadherin's presence was evident in the perinuclear area as well as in the cytoplasm. In the revertant MCF-7/T cells, E-cadherin and β-catenin were also readily detectable at the cell adhesion areas with well defined staining (Figure 7A; panels C and D, respectively). Further detailed analysis of the organization of these two molecules in the revertant cells revealed significant differences from the parental MCF-7 cells.

While both parental MCF-7 and MCF-7/T cells contained E-cadherin and β-catenin at cell-cell junctions, their association with the detergent insoluble fraction was different between the cell types. The samples were stained with respective antibodies, visualized using a Zeiss fluorescence microscope and photographed for different exposures as described in the Materials and Methods section. The samples were exposed to accumulate different number of frames, without any other adjustments to either black level or brightness settings. It was found that MCF-7 cells had significantly lower detergent-resistant E-cadherin and β-catenin at the cell-cell junctions (Figure 7B, panels a, b) vis-à-vis MCF-7/T cells (Figure 7B, panels c, d). When the samples were photographed for the same number of frames, the intensity of either E-cadherin or β-catenin at the cell-cell junctions was higher in MCF-7/T cells. MCF-7/T cells consistently retained higher amounts of E-cadherin and β-catenin at the cell-cell junctions than in MCF-7 cells. Multiple areas of both the cell types were examined at more than two gate settings. These data suggest that in the revertant cells, E-cadherin and β-catenin are more tightly linked to the cytoskeleton, which is in line with the immunoblotting results (Fig. 6). Under these conditions, α- and γ-catenins, however, were found to be at comparable levels in MCF-7 and MCF-7/T cells (data not shown).

Discussion:

It is now widely recognized that normal functioning of cytoskeleton is essential for maintaining normal growth and differentiation. For example, actin microfilaments are important determinants of cell morphology, cell motility, cell polarity and cell division. In addition, reorganization of actin microfilaments occurs in response to activation of integrins and it is likely to play an important role in 'inside out' signaling (Schwartz, 1997). The attachment of cadherin-catenin complexes to microfilaments is known to strengthen cell-cell adhesion and contribute to tissue integrity. Furthermore, more recent data suggest that, many signaling molecules are linked to microfilaments (McCartney *et al.*, 1999). Transformed cells generally lack well-defined microfilaments which, besides the cause for loss of normal morphology, are important contributing factors for the metastatic behavior (Hirohashi, 1998).

Several lines of evidence presented herein support the thesis that TM1 is a suppressor of the malignant growth phenotype of breast cancer cells: (i) restoration of TM1 expression results in the growth of MCF-7 cells as tighter colonies with a more glandular morphology, while the parental and wild type MCF-7 cells grew as more scattered colonies; (ii) TM1 expression leads to significantly decreased growth in monolayer cultures, and; (iii) TM1 expression completely abolishes the anchorage independent growth of MCF7 cells. Earlier work from this laboratory has demonstrated that TM1 reverts the transformed phenotype of *Ki-ras* or *v-src*-transformed fibroblasts. Taken together, these findings are consistent with the hypothesis that TM1 is a general suppressor of malignant transformation.

Cadherin-catenins complexes mediate cell-cell adhesion and a number of studies have demonstrated that the integrity and functioning of these cell adhesion complexes are disrupted in many types of cancers, including those originating in breast (Asgeirsson *et al.*, 2000; Bukholm *et*

al., 1998). E-cadherin, often referred to as a metastasis suppressor, is generally expressed either at low levels (Mareel *et al.*, 1997; Sommers, 1996) or is mutated in some breast cancer specimens (Huiping *et al.*, 1999). A variety of mechanisms contribute to silencing of E-cadherin gene (Graff *et al.*, 2000; Hajra *et al.*, 1999; Ji *et al.*, 1997). The dysfunctional E-cadherin-catenin complexes are often correlated with increased cell motility and metastasis. It is generally accepted that the interactions of cadherin-catenin complexes with the cytoskeleton are important in maintaining normal adhesion via cadherins (Gumbiner, 2000; Yap *et al.*, 1997)

Unlike some other breast carcinoma cells, MCF-7 cells express readily detectable and comparable levels of the components of the cell adhesion molecules (Sommers *et al.*, 1994). In TM1 expressing revertants of MCF-7 cells, while there appear to be no qualitative differences, E-cadherin and the catenins are more tightly coupled to cytoskeleton as evident from differential extractability of these proteins. This is likely to be facilitated by improved microfilamental organization induced by TM1 expression (Figure 3B). Enhanced detergent solubility of E-cadherin and β-catenin is more frequently observed in transformed cells, which may result in the assembly of defective adhesion junctions. In TM1-expressing revertant cells, E-cadherin and β-catenin are more tightly associated with the cytoskeleton, as evidenced by immunoblotting studies (Fig.7) and immunofluorescence experiments. Consistent with these data, it was reported that ras-transformation of breast epithelial cells does not change the expression of E-cadherin or β-catenin, but results the increased detergent solubility of these molecules (Kinch *et al.*, 1995). Increased binding to cytoskeletal elements is also reflected in the localization of E-cadherin and β-catenin to cell-cell boundaries of the revertant cells. It is likely that the improved/TM1-induced microfilaments may provide the necessary support for clustering and stability of the cadherins

(Yap *et al.*, 1997). This may also be evident in the formation of tighter and more organized morphology of MCF-7/T cells (Fig 3A).

The Rho family of GTPases, in addition to reorganizing microfilaments, regulate cell-cell and cell-substratum adhesion. It is proposed GTP-bound Cdc-42 and Rac proteins bind to IQGAP and disrupt IQGAP-β-catenin interaction to promote stable cadherin-catenin complexes (Kaibuchi *et al.*, 1999). Additionally, p-21-activated kinase-1 (PAK-1) has been implicated in the reorganization of microfilaments and invasiveness of breast cancer cells (Adam *et al.*, 1998; Adam *et al.*, 2000).

In addition to TM1, other cytoskeletal proteins such as gelsolin, α-actinin, vinculin and profilin1 have been shown to be suppressors of the transformed phenotype. Gelsolin and profiling bind to phosphotidylinositol 4,5 bis phosphate (PIP2), which is an important second messenger in the intracellular signaling networks. Vinculin has been shown to substitute for α-catenin in cadherin-catenin complexes in a different breast carcinoma cell line, where α-catenin was mutated (Hazan *et al.*, 1997). Furthermore, upon activation of the EGF receptor, α-catenin and vinculin dissociate from E-cadherin-catenin complex coupled with a robust tyrosine phosphorylation of β- catenin, γ-catenins and p120 ^{cas} suggesting that the EGF receptor regulates the linkage of microfilaments to adherens junctions (Hazan *et al.*, 1997). EGF receptor activation, however, does not alter the levels, either of E-cadherin, or the catenins. Similarly, phosphorylation in ser/thr residues of the cytoplasmic portion of E-cadherin by casein kinase II and GSK-3β enhances binding to β-catenin (Lickert *et al.*, 2000). Therefore, altered post-translational modifications such as phosphorylation, may be critical in facilitating a tighter linkage of cadherin-and the catenins to cytoskeleton in the TM1-mediated reversion of MCF-7 cells.

Since TM1 binds to and stabilizes actin microfilaments, TM1-induced microfilaments may provide the dynamic support for the reorganization of cadherin-catenin complexes. While the exact mechanisms of TM1-induced reorganization of cadherin-catenin complexes and suppression of the transformed phenotype of breast cancer cells are unclear at present, our recent studies have indicated that TM1-induced cytoskeletal reorganization is mediated by Rho kinase (Shah *et al.*, 1998). Given the multitude of roles of the actin cytoskeleton, it is likely that TM1-induced cytoskeleton will critical in TM1-induced tumor suppression.

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References:

Adam, L., Vadlamudi, R., Kondapaka, S.B., Chernoff, J., Mendelsohn, J. & Kumar, R. (1998). *J. Biol. Chem.*, **273,** 28238-28246.

Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J. & Kumar, R. (2000). *J. Biol. Chem.*, **275,** 12041-12050.

Asgeirsson, K.S., JG, J.o., Tryggvad:ottir, L., Olafsd:ottir, K., Sigurgeirsd:ottir, J.R., Ingvarsson, S. & Ogmundsd:ottir, H.M. (2000). *Eur J. Cancer*, **36,** 1098-1106.

Bhattacharya, B., Prasad, G.L., Valverius, E.M., Salomon, D.S. & Cooper, H.L. (1990). *Cancer Res.*, **50,** 2105-2112.

Bissell, M.J., Weaver, V.M., Lelievre, S.A., Wang, F., Petersen, O.W. & Schmeichel, K.L. (1999). *Cancer Res.*, **59,** 1757-1763s; discussion 1763s-1764s.

Braverman, R.H., Cooper, H.L., Lee, H.S. & Prasad, G.L. (1996). *Oncogene*, **13,** 537-545.

Bukholm, I.K., Nesland, J.M., Karesen, R., Jacobsen, U. & Borresen-Dale, A.L. (1998). *J. Pathol.*, **185,** 262-266.

Cooper, H.L., Bhattacharya, B., Bassin, R.H. & Salomon, D.S. (1987). *Cancer Res.*, **47,** 4493-4500.

Cooper, H.L., Feuerstein, N., Noda, M. & Bassin, R.H. (1985). *Mol. Cell. Biol.*, **5,** 972-983.

Cutler, M.L., Bassin, R.H., Zanoni, L. & Talbot, N. (1992). *Mol. Cell. Biol.*, **12,** 3750-3756.

Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B. & Herman, J.G. (2000). *J. Biol. Chem.*, **275,** 2727-2732.

Gumbiner, B.M. (2000). *J. Cell Biol.*, **148,** 399-404.

Hajra, K.M., Ji, X. & Fearon, E.R. (1999). *Oncogene*, **18,** 7274-7279.

Hazan, R.B., Kang, L., Roe, S., Borgen, P.I. & Rimm, D.L. (1997). *J. Biol. Chem.*, **272,** 32448-32453.

Hirohashi, S. (1998). *American Journal of Pathology*, **153,** 333-339.

Huiping, C., Sigurgeirsdottir, J.R., Jonasson, J.G., Eiriksdottir, G., Johannsdottir, J.T., Egilsson, V. & Ingvarsson, S. (1999). *British Journal of Cancer*, **81,** 1103-1110.

Ishikawa, R., Yamashiro, S. & Matsumura, F. (1989a). *J. Biol. Chem.*, **264,** 16764-16770.

Ishikawa, R., Yamashiro, S. & Matsumura, F. (1989b). *J. Biol. Chem.*, **264,** 7490-7497.

Ji, X., Woodard, A.S., Rimm, D.L. & Fearon, E.R. (1997). *Cell Growth. Diffn.*, **8,** 773-778.

Kaibuchi, K., Kuroda, S., Fukata, M. & Nakagawa, M. (1999). *Curr. Opin. Cell Biol.*, **11,** 591-596.

Kamarainen, M., Seppala, M., Virtanen, I. & Andersson, L.C. (1997). *Laboratory Investigation*, **77,** 565-573.

Kinch, M.S., Clark, G.J., Der, C.J. & Burridge, K. (1995). *J. Cell Biol.*, **130,** 461-471.

Kinzler, K.W. & Vogelstein, B. (1997). *Nature*, **386,** 761, 763.

Lickert, H., Bauer, A., Kemler, R. & Stappert, J. (2000). *J. Biol. Chem.*, **275,** 5090-5095.

Lin, J.J., Warren, K.S., Wamboldt, D.D., Wang, T. & Lin, J.L. (1997). *Intl. Rev. Cytol.*, **170,** 1-38.

Mareel, M., Boterberg, T., Noe, V., Van Hoorde, L., Vermeulen, S., Bruyneel, E. & Bracke, M. (1997). *J. Cell Phys.*, **173,** 271-274.

Matsumura, F., Lin, J.J., Yamashiro-Matsumura, S., Thomas, G.P. & Topp, W.C. (1983). *J. Biol. Chem.*, **258,** 13954-13964.

McCartney, B.M., Dierick, H.A., Kirkpatrick, C., Moline, M.M., Baas, A., Peifer, M. & Bejsovec, A. (1999). *J. Cell Biol.*, **146,** 1303-1318.

Oldham, S.M., Clark, G.J., Gangarosa, L.M., Coffey, R.J., Jr. & Der, C.J. (1996). *Proc. Natl. Acad. Sci. USA.*, **93,** 6924-6928.

Pawlak, G. & Helfman, D.M. (2001). *Curr. Opin. Gen. Dev*, **11,** 41-47.

Pittenger, M.F., Kazzaz, J.A. & Helfman, D.M. (1994). *Curr. Opin. Cell Biol.*, **6,** 96-104.

Polakis, P. (1999). *Curr. Opin. Gen. Dev*, **9,** 15-21.

Polakis, P., Hart, M. & Rubinfeld, B. (1999). *Advances in Experimental Medicine & Biology*, **470,** 23-32.

Prasad, G.L., E.M.Valverius, E.McDuffie & H.L.Cooper. (1992). *Cell Growth. Diffn.*, **3,** 507-513.

Prasad, G.L., Fuldner, R.A., Braverman, R., McDuffie, E. & Cooper, H.L. (1994). *Eur. J. Biochem.*, **224,** 1-10.

Prasad, G.L., Fuldner, R.A. & Cooper, H.L. (1993). *Proc. Natl. Acad. Sci. USA.*, **90,** 7039-7043.

Prasad, G.L., Masuelli, L., Raj, M.H. & Harindranath, N. (1999). *Oncogene*, **18,** 2027-2031.

Prasad, G.L., P.S.Meissner, D.Sheer & H.L.Cooper. (1991). *Biochem. Biophys. Res. Commun.*, **177,** 1068-1075.

Schafer, R. (1994). *Reviews of Physiology Biochemistry & Pharmacology*, **124,** 29-92.

Schwartz, K., Richards, K. & Botstein, D. (1997). *Mol. Biol Cell*, **8,** 2677-2691.

Schwartz, M.A. (1997). *J. Cell Biol.*, **139,** 575-578.

Sers, C., Emmenegger, U., Husmann, K., Bucher, K., Andres, A.C. & Schafer, R. (1997). *J. Cell Biol.*, **136,** 935-944.

Shah, V., Bharadwaj, S., Kaibuchi, K. & Prasad, G.L. (2001). *Oncogene*, **20,** 2112-2121.

Shah, V., Braverman, R. & Prasad, G.L. (1998). *Somatic Cell and Mol. Gen*, **24,** 273-280.

Sommers, C.L. (1996). *J. Mammary Gland Biol. & Neoplasia*, **1,** 219-229.

Sommers, C.L., Byers, S.W., Thompson, E.W., Torri, J.A. & Gelmann, E.P. (1994). *Breast. Cancer. Res. Treat.*, **31,** 325-335.

Yamashiro, S., Yamakita, Y., Ono, S. & Matsumura, F. (1998). *Mol. Biol Cell*, **9,** 993-1006.

Yap, A.S., Brieher, W.M. & Gumbiner, B.M. (1997). *Ann. Rev. Cell Dev. Biol.*, **13,** 119-146.

Figure Legends:

Figure 1. TM expression in mammary epithelial cells: A. Two dimensional gel analysis of TM expression in MCF10A cells was performed as described in Materials and Methods. TM1, TM38 and TM32 are identified.

B. TM expression in normal and malignant breast cells. The cell lines used are indicated. DT/TM1 and DT/TM1-TM2 cells were used as positive controls for TM expression. These are TM1-induced revertants of ras-transformed fibroblasts expressing either TM1 alone or both TM1 and TM2 (Shah *et al.*, 1998). TM2 migrates as a distinct band below TM1, which is evident in DT/TM1-TM2 cells (top panel). The cell lysates were probed with either a polyclonal antiserum that reacts to multiple TMs (top panel), an anti-peptide antibody that reacts to TM1 specifically (middle panel), or with anti tubulin antibody for load controls (bottom panel).

Figure 2. Tropomyosin Expression: A. Northern blotting: Total cellular RNAs were probed with a full length cDNA encoding TM1 at high stringency. Transduced TM1 is expressed as a 2.0kb mRNA, while the endogenous TM1 is synthesized as a 1.1kb RNA. The parental MCF-7 cells lack the mRNA encoding TM1. β-actin was used for load controls (bottom panel).

B. Two dimensional gel analyses of TM expression: Total cell lysates were prepared from pulse labeled cells and analyzed by two-dimensional gels. TM1 was identified in the right panel.

Figure 3. A. Morphology of TM1 expressing MCF-7 cells: Monolayers of MCF-7 and MCF-7/T cells were stained with H & E and photographed using an Olympus B20 microscope with 2x objective.

B. TM1 associates with microfilaments: MCF-7 (A-C) and MCF-7/T (D-F) cells were immunostained with TM antiserum (A, D) followed by binding to FITC conjugated anti-rabbit antibody (green) and Texas red conjugated phalloidin (B, E; red). Merged images (C, F) are presented. The samples were viewed using a confocal microscope.

Figure 4. TM1 expression decreases growth of MCF-7 cells: Cells were cultured under normal growth medium (4A), in charcoal stripped medium (estrogen deprivation), or in charcoal stripped serum supplemented with 100nM estradiol. The growth of MCF-7 and MCF-7/V (Figure 4B), the three MCF-7/T cell lines (Figure 4C) and under conditions of estrogen deprivation and supplementation is depicted. Error bars indicate standard deviation. The revertant cells grew significantly slower than the parental or vector control MCF-7 cells in normal serum, in the absence or under estrogen supplementation.

Figure 5. TM1 suppresses anchorage independent growth: Cells were plated in soft agar as described in materials and methods. At the end of the culture, they were stained with nitroblue tetrazolium, photographed (A) and the number of colonies formed with each cell line is shown (B). Error bars indicate standard deviation.

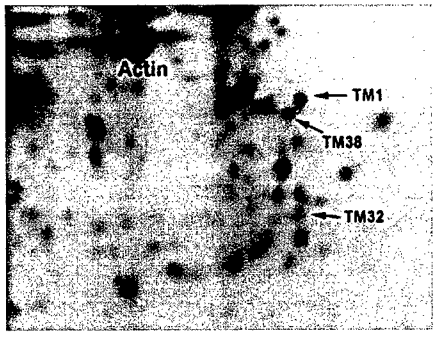
Figure 6. E-Cadherin-catenin complex is more tightly associated in MCF-7/T revertant cells: Cytoplasmic lysates (A), and RIPA lysates (B) of the indicated cells were analyzed for expression of E-cadherin and the catenins by immunoblotting. Note that in the RIPA extracts, E-cadherin and the catenins are expressed at comparable levels between the transformed and the revertant lines of MCF-7 cells.

Figure 7A. Organization of E-cadherin and β -catenin: MCF-7 (panels A, B) and MCF-7/T (panels C, D) cells were stained with either E-cadherin (panels A, C) or β-catenin (panels B, D) and examined by immunocytochemistry. In both cell types, the cell adhesion molecules are found at the cell-cell junctions.

Figure 7B. E-cadherin and β-catenin are tightly associated at the cell-cell junctions of MCF-7/T Cells: MCF-7 (panels a,b) and MCF-7/T (panels c,d) cells were stained with either E-cadherin (panels a,c) or β-catenin (panels b,d). The samples were exposed at different gate settings to accumulate different numbers of frames, which is dependent on the intensity of the signal. E-cadherin stained samples were photographed at 16 frames, and β-catenin at 8 frames. Images at other higher or lower settings are not shown.

Figure 1

A.


Actin
TM1
TM38
TM32

B.

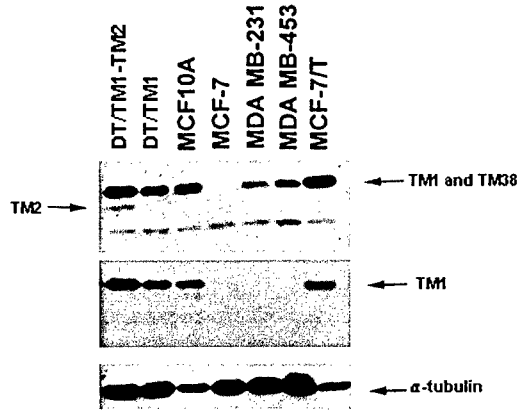
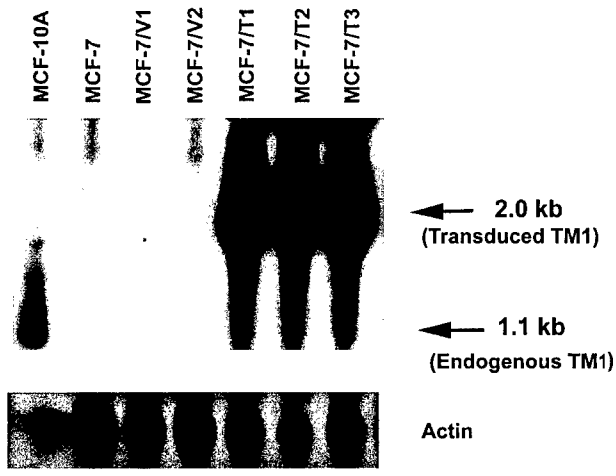

DT/TM1-TM2
DT/TM1
MCF10A
MCF-7
MDA MB-231
MDA MB-453
MCF-7/T
TM2
TM1 and TM38
TM1
α-tubulin

Figure 2

A.


MCF-10A
MCF-7
MCF-7/V1
MCF-7/V2
MCF-7/T1
MCF-7/T2
MCF-7/T3
2.0 kb
(Transduced TM1)
1.1 kb
(Endogenous TM1)
Actin

B.

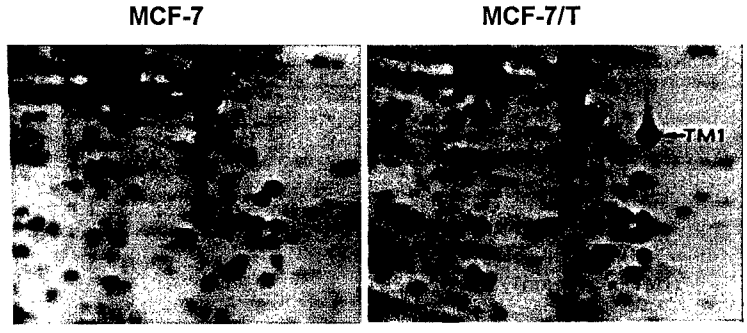
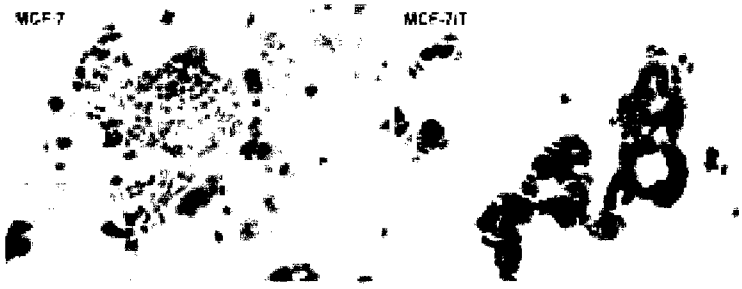

MCF-7
MCF-7/T
TM1

Figure 3

A.



B.

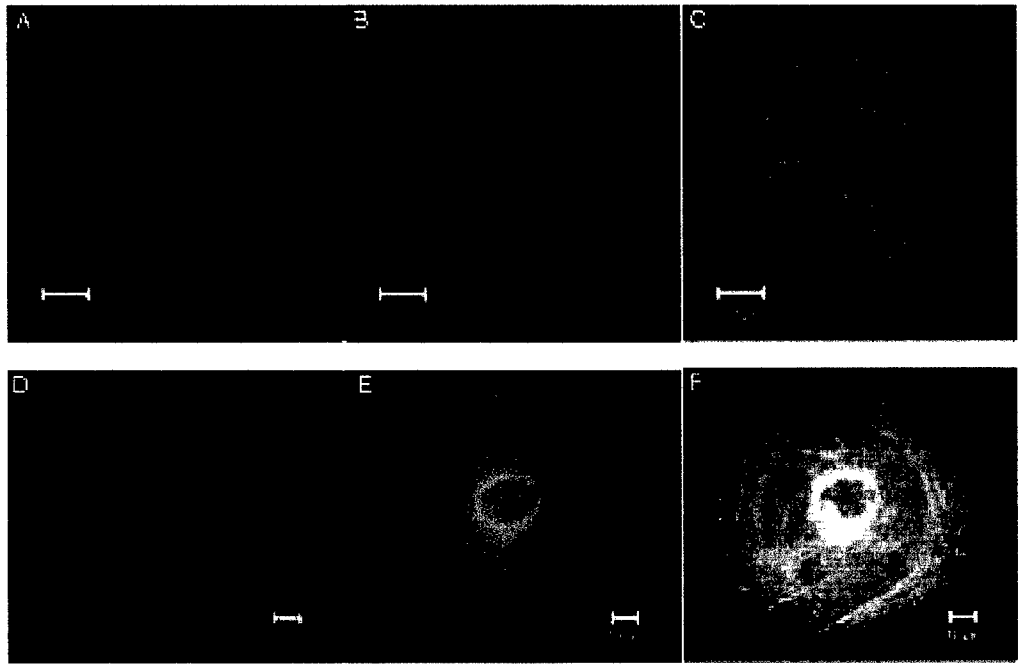


Figure 4

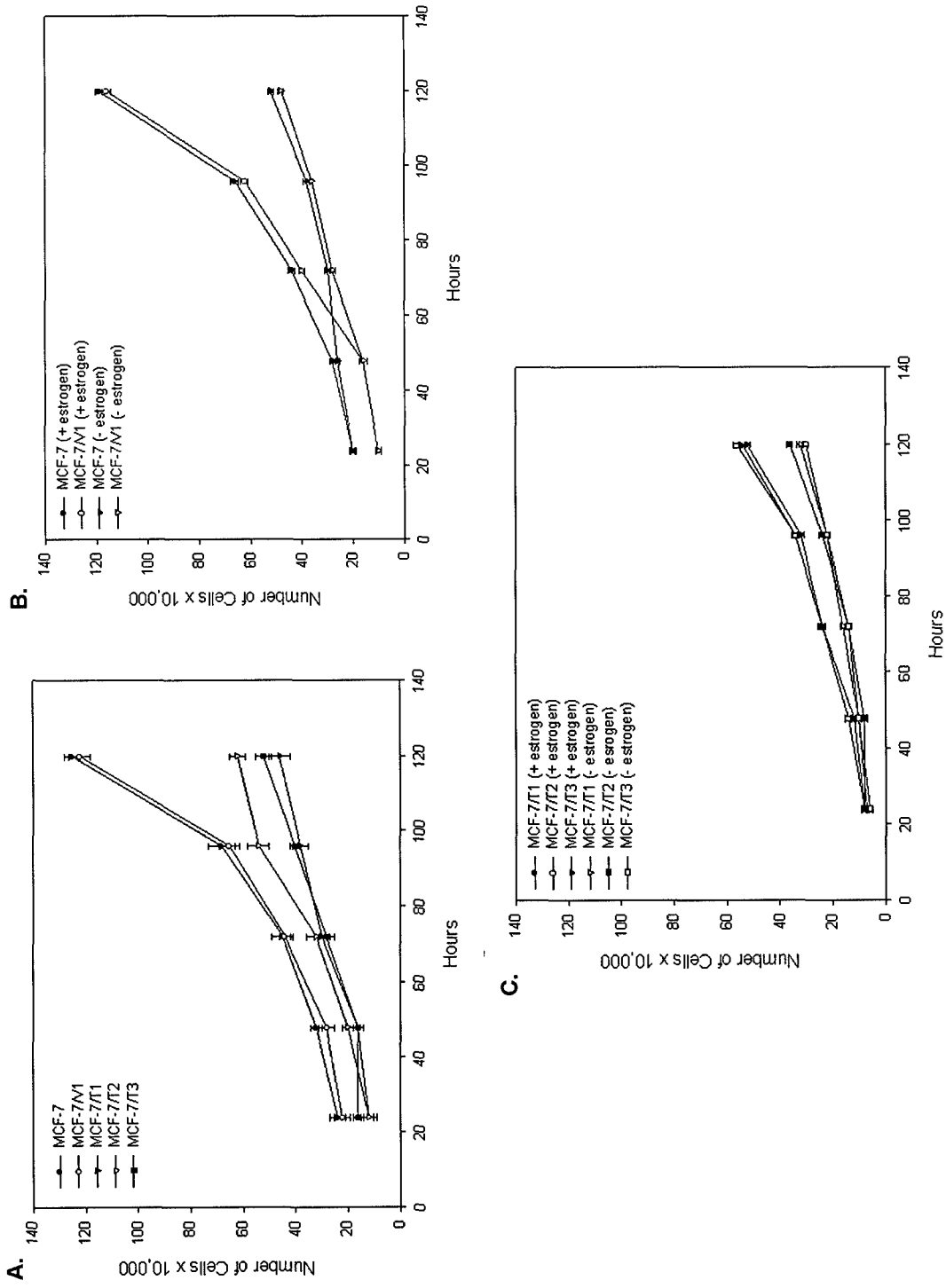
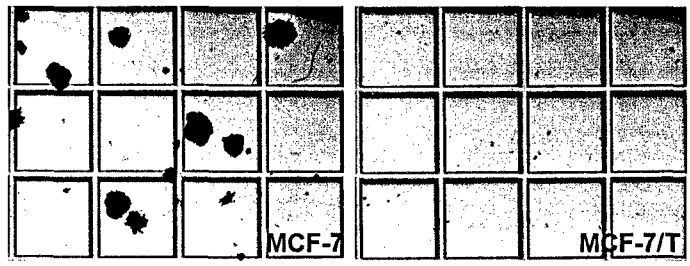

A.
Number of Cells x 10,000
Hours
MCF-7
MCF-7/V1
MCF-7/T1
MCF-7/T2
MCF-7/T3
B.
Number of Cells x 10,000
Hours
MCF-7 (+ estrogen)
MCF-7/V1 (+ estrogen)
MCF-7 (- estrogen)
MCF-7/V1 (- estrogen)
C.
Number of Cells x 10,000
Hours
MCF-7/T1 (+ estrogen)
MCF-7/T2 (+ estrogen)
MCF-7/T3 (+ estrogen)
MCF-7/T1 (- estrogen)
MCF-7/T2 (- estrogen)
MCF-7/T3 (- estrogen)

Figure 5

A.


MCF-7
MCF-7/T

B.

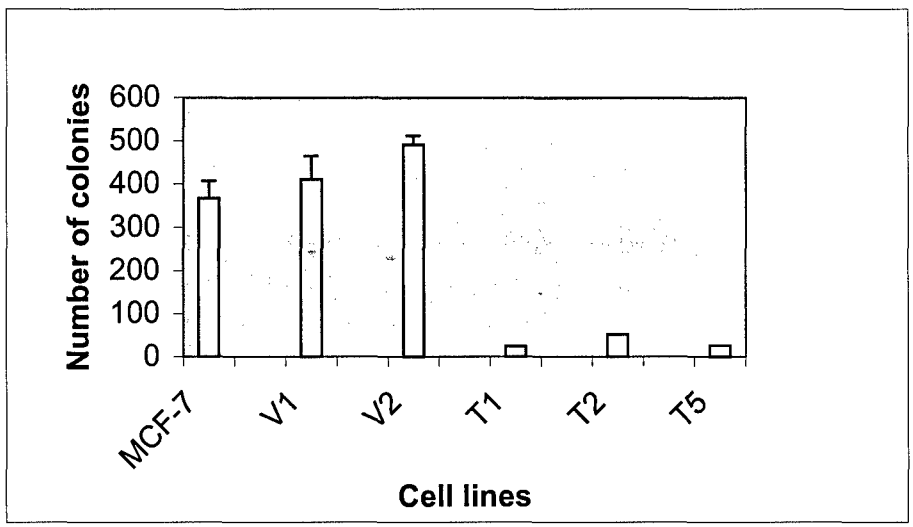

Number of colonies
600
500
400
300
200
100
0
MCF-7
V1
V2
T1
T2
T5
Cell lines

Figure 6

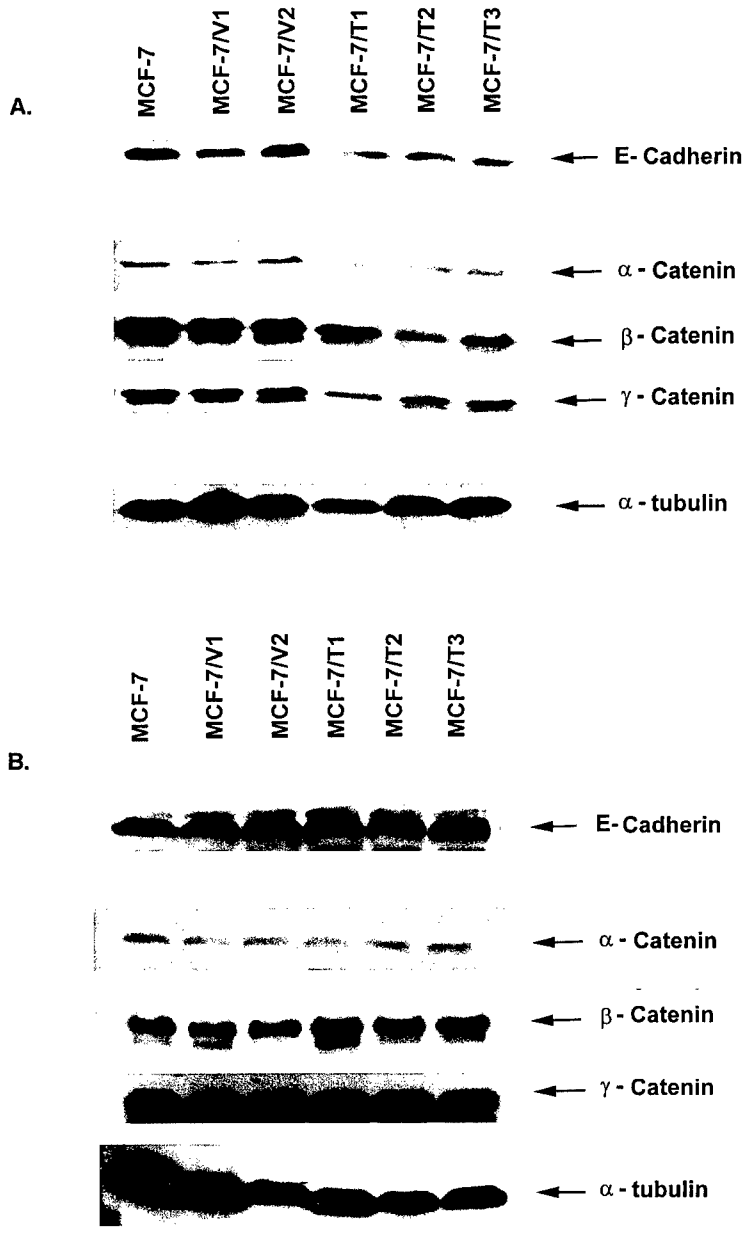

A.
MCF-7
MCF-7/V1
MCF-7/V2
MCF-7/T1
MCF-7/T2
MCF-7/T3
E- Cadherin
α - Catenin
β - Catenin
γ - Catenin
α - tubulin
B.
MCF-7
MCF-7/V1
MCF-7/V2
MCF-7/T1
MCF-7/T2
MCF-7/T3
E- Cadherin
α - Catenin
β - Catenin
γ - Catenin
α - tubulin

Figure 7A

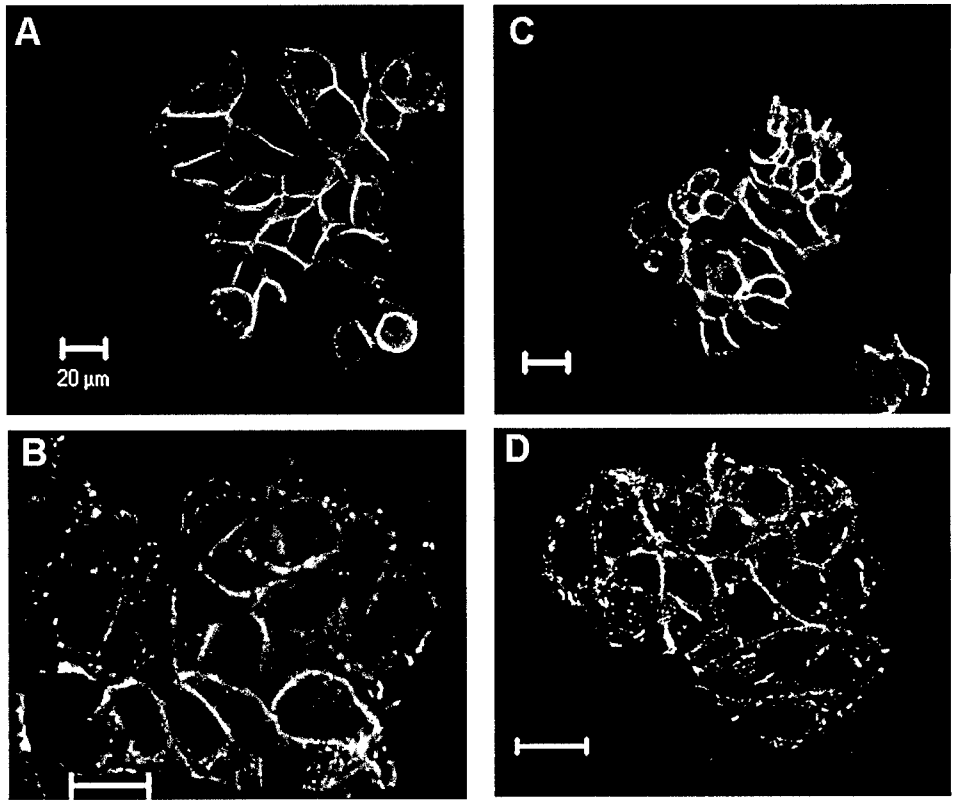
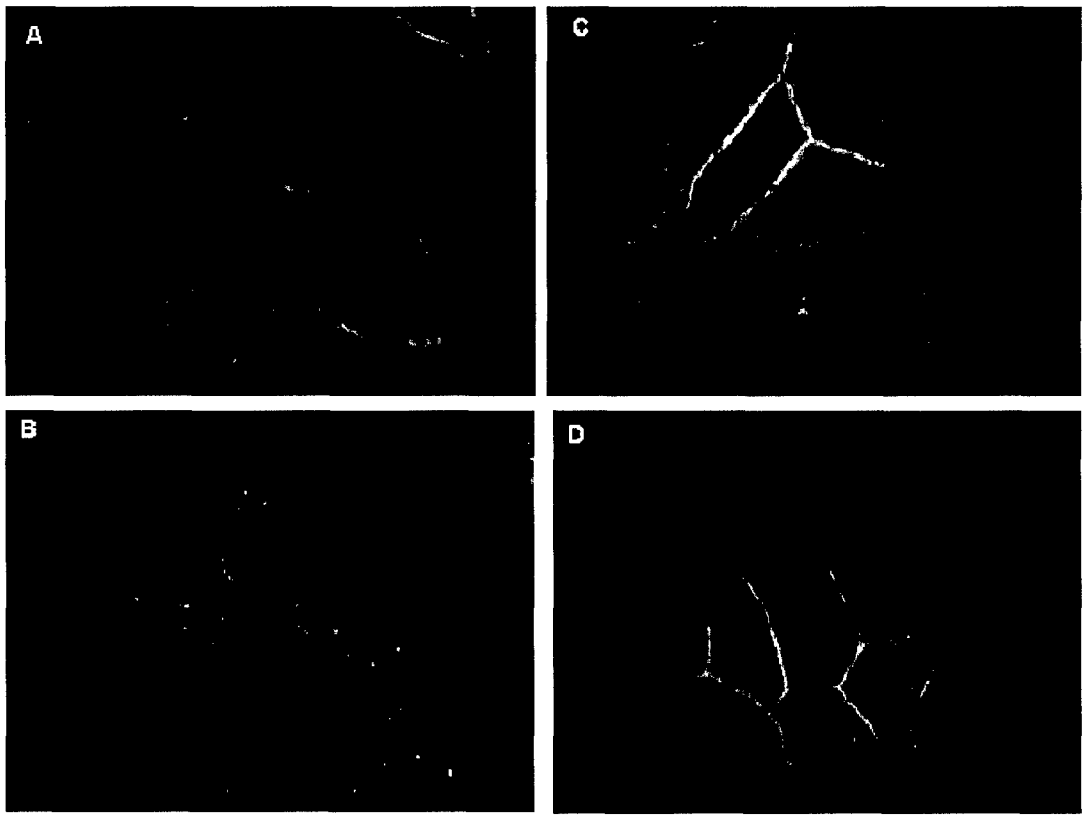


Figure 7B



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Cytoskeletal organization in tropomyosin-mediated reversion of *ras*-transformation: Evidence for Rho kinase pathway

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Tropomyosin (TM) family of cytoskeletal proteins is implicated in stabilizing actin microfilaments. Many TM isoforms, including tropomyosin-1 (TM1), are down-regulated in transformed cells. Previously we demonstrated that TM1 is a suppressor of the malignant transformation, and that TM1 reorganizes microfilaments in the transformed cells. To investigate how TM1 induces microfilament organization in transformed cells, we utilized *ras*-transformed NIH3T3 (DT) cells, and those transduced to express TM1, and/or TM2. Enhanced expression of TM1 alone, but not TM2, results in re-emergence of microfilaments; TM1, together with TM2 remarkably improves microfilament architecture. TM1 induced cytoskeletal reorganization involves an enhanced expression of caldesmon, but not vinculin, α-actinin, or gelsolin. In addition, TM1-induced cytoskeletal reorganization and the revertant phenotype appears to involve re-activation of RhoA controlled pathways in DT cells. RhoA expression, which is suppressed in DT cells, is significantly increased in TM1-expressing cells, without detectable changes in the expression of Rac or Cdc42. Furthermore, expression of a dominant negative Rho kinase, or treatment with Y-27632 disassembled microfilaments in normal NIH3T3 and in TM1 expressing cells. These data suggest that reactivation of Rho kinase directed pathways are critical for TM1-mediated microfilament assemblies. *Oncogene* (2001) **20,** 2112–2121.

Keywords: tropomyosin; cytoskeleton; Rho kinase

Introduction

Expression of many cytoskeletal proteins is down-regulated during neoplastic transformation, resulting in an altered cell morphology, reorganization of cytoarchitecture, cell motility, and possibly contributing to changes in gene expression by modulating intracellular signaling pathways (Button *et al.*, 1995; Janmey and Chaponnier, 1995). For example, many microfilament associated proteins such as α-actinin, vinculin, gelsolin and tropomyosins (TMs) are suppressed to varying degrees in many transformed cells (Ben-Ze'ev, 1997). Furthermore, restoration of these proteins inhibits the malignant phenotype of many different experimentally transformed cell lines, underscoring the pivotal role of cytoskeletal organization in maintaining a normal phenotype (Ayscough, 1998; Janmey and Chaponnier, 1995). Our laboratory has been interested in understanding the role of cytoskeletal proteins, in particular that of tropomyosins, in malignant transformation.

Tropomyosin (TM) family comprises of 5–7 different closely related isoforms, whose expression is altered in many transformed cells (Lin *et al.*, 1997; Pittenger *et al.*, 1994). For example, suppression of high M_r TMs, *viz.*, isoforms 1 and 2 (TM1 and TM2, respectively), is nearly universal in all the transformed cell lines tested, while elevated levels of the low M_r species such as TM4 and TM5 are reported in some malignant cell types (Bhattacharya *et al.*, 1990; Cooper *et al.*, 1985, 1987; Hendricks and Weintraub, 1981; Leavitt *et al.*, 1986; Matsumura *et al.*, 1983). Although all TMs bind to actin with varying binding affinities, the precise function of each of the isoforms remains largely unknown. Other actin binding proteins, such as caldesmon, also influence TM binding to actin to varying degrees (Pittenger *et al.*, 1995). Our efforts to elucidate the causal relationship between tropomyosin expression and cell phenotype have uncovered significant fundamental differences in the roles of TM isoforms in cell physiology (Braverman *et al.*, 1996; Prasad *et al.*, 1993; Shah *et al.*, 1998).

In DT (v-*ki-ras*-transformed NIH3T3) cells, TM1 expression is suppressed to 50% levels found in normal fibroblasts, but TM2 levels are essentially undetectable (Cooper *et al.*, 1985; Prasad *et al.*, 1993). DT cells are spindle shaped with no defined microfilament structures and are highly malignant. Transduction of DT cells with a cDNA encoding TM1 protein results in reorganization of cytoskeleton with well-defined microfilaments. More significantly, elevated TM1 levels inhibited the transformed phenotype of DT cells, culminating in a stable revertant phenotype (Braverman *et al.*, 1996; Prasad *et al.*, 1993). In addition, TM1 also suppresses v-*src*-induced transformed phenotype, suggesting that TM1 may be a general suppressor of transformation that belongs to class II tumor suppressors (Prasad *et al.*, 1999). On the contrary, restoration

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of expression of TM2 protein did not improve the cytoskeleton, and the cells remained transformed, resembling DT cells (Braverman *et al.*, 1996). Co-expression of TM2 with TM1, however, dramatically improved microfilaments, similar to those in normal NIH3T3 cells: DT cells expressing TM1 and TM2 exhibit a revertant phenotype (Shah *et al.*, 1998). It is intriguing that TM1, an actin binding protein reorganizes the cytoskeleton and restores the normal growth phenotype, while the other closely related TMs despite their ability to bind to actin are unable to modulate cytoarchitecture and growth phenotypes (Braverman *et al.*, 1996; Pittenger *et al.*, 1994). While TM1 alone is able to organize the other cytoskeletal proteins into microfilaments to an appreciable degree, together with TM2 it restores the cytoarchitecture to the extent found in normal cells.

The restored cytoskeleton may be important for the anti-oncogenic effects of TM1. In the transformed cells, along with TMs, the expression myosin light chain and many other key microfilament proteins are also suppressed (Ben-Ze'ev, 1997; Janmey and Chaponnier, 1995; Kumar and Chang, 1992). To elucidate the nature of TM1-induced formation of microfilaments, we have evaluated the changes in the expression of other key cytoskeletal proteins and tested the stability of TM1-induced cytoskeleton in the DT cell system described above. Reorganization of cytoskeleton, arising from the forced expression of TM1, will have to involve recruitment of some of the other cytoskeletal proteins from the existing pool; alternatively, through yet unknown mechanisms, TM1 might contribute to an increase in their synthesis. Our results, for the first time, show that TM1 reorganizes cytoskeleton via both of these mechanisms. We also provide evidence for the involvement of Rho-regulated pathways, in the emergence of microfilaments. These pathways could play a critical role in modulating gene expression, to culminate in a revertant phenotype.

Results

Expression of cytoskeletal proteins in TM1 mediated reversion

To investigate the role of TMs in cellular transformation, we generated several cell lines of DT expressing TM1 (DT/TM1 cells), TM2 (DT/TM2 cells) or both the TMs (DT/TM1-TM2 cells) by introduction of cDNAs encoding TM1, TM2 or both the TMs, respectively (Table 1; Figure 1) (Braverman *et al.*, 1996; Prasad *et al.*, 1991, 1993; Shah *et al.*, 1998). In transformed cells, in addition to TMs, many other proteins including those associated with microfilaments are also suppressed. For example, expression of gelsolin, vinculin, α-actinin and caldesmon is down-regulated in many transformed cells and tumors (Asch *et al.*, 1999; Ben-Ze'ev, 1997; Button *et al.*, 1995; Janmey and Chaponnier, 1995; Mielnicki *et al.*, 1999). Given the high degree of the malignant nature of DT cells, these changes are expected to be extensive. It should be noted that TM1 and TM2 share extensive homology, yet only TM1 is capable of reorganizing microfilaments and suppressing the Ras-induced transformed phenotype (Braverman *et al.*, 1996; Shah *et al.*, 1998). The properties of microfilaments induced by TM1 in DT cells could significantly differ because the expression of many components is likely to remain repressed due to the continued oncogenic action of Ras. To understand the molecular basis of reorganization of cytoskeleton in DT/TM1 and DT/TM1-TM2 cells, expression of some of the key microfilament-associated proteins was investigated in normal NIH3T3, DT, DT cells transduced with an empty pBNC vector (DT/V), DT/TM1, DT/TM2 and DT/TM1-TM2 cells.

Vinculin expression was significantly lower in DT and the derived cell lines compared to normal fibroblasts (Figure 2a), a finding consistent with the published data (Rodriguez Fernandez *et al.*, 1992). Immunoprecipitation followed by Western blotting with a specific antibody revealed that expression of vinculin remained low in the cell lines derived from DT, regardless of the phenotype. Then we investigated whether TM1 expression modulates distribution of vinculin in focal adhesions by confocal microscopy (Figure 2b). The samples were stained with anti-vinculin antibody optically, sectioned with a confocal microscope and the composite images are presented. In normal NIH3T3 cells, vinculin was well spread through out the cell body and organized as dense particles, indicative of focal adhesions. In DT cells, where a 25–30% reduction in vinculin levels is noted, a different organization, consistent with the lack of microfilaments, was observed: vinculin is mostly concentrated to the perinuclear area. In DT/TM1 cells, the vinculin staining is more spread out. In cells expressing both TM1 and TM2, however, vinculin was spread more evenly in the cell body, with the presence of 'speckled' appearance indicating a better organization. The samples were also stained for the presence of actin filaments by Texas-Red conjugated phalloidin (data not shown).

Caldesmon, which regulates binding of TMs to microfilaments, is also down regulated in transformed cells. Data of Figure 3a show that expression of caldesmon mRNA is suppressed 40% in DT, DT/V and DT/TM2 cells. However, in DT/TM1 (90%) and DT/TM1-TM2 (≈100%) cells caldesmon mRNA expression is nearly restored to the levels found in normal fibroblasts. Caldesmon protein levels were also enhanced in the revertant DT/TM1 and DT/TM1-TM2 cells, compared to the parental DT cells, as determined by immunoblotting (Figure 3b).

On the other hand, there are no consistent changes in the expression of α-actinin in NIH3T3, DT and the derived cell lines (data not shown). In normal fibroblasts, transformed and the revertant cells, α-actinin levels did not change appreciably. However, in cell lines expressing TM2, there appears to be a modest decrease in α-actinin levels. Western blotting with anti-

Table 1 DT cell model system

Cell line	*Derived from*	*Generated by*	*Growth phenotype*	*TM1 expression*	*TM2 expression*	*TM3 expression*
NIH3T3 cells	Parent cells	Parent cells	Normal	+ + normal	+ + normal	+ + normal
DT	NIH3T3	Transformation with two copies of *v-Ki-ras*	Malignant	+ (50% decrease)	– (not detectable)	– (not detectable)
DT/TM1	DT	Transduction with TM1 cDNA	Revertant	⩾ + + (enhanced)	– (not detectable)	– (not detectable)
DT/TM2	DT	Transfection with TM2 cDNA	Malignant	+ (same as in DT)	⩾ + + (enhanced)	– (not detectable)
DT/TM1-TM2	DT/TM1	Transfection with TM2 cDNA	Revertant	⩾ + + (enhanced)	⩾ + + (enhanced)	– (not detectable)

Normal NIH3T3 cells have been transformed by two coies of *v-ki-ras* to generate DT cells, which express decreased TM1, and no detectable TM2 or TM3. Transduction of DT cells with a recombinant retrovirus to express high levels of TM1 reverts DT cells to normal phenotype with a well spread morphology and microfilaments (DT-TM1). Restoration of TM2, however, does not alter the cell morphology or the phenotype (DT-TM2). But co-expression of TM2 remarkably improves the microfilament architecture of DT/TM1 cells (DT/TM1-TM2). Growth phenotypes of the cell lines is indicated

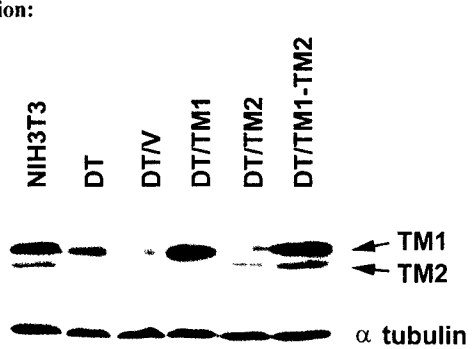

Figure 1 TM expression in DT cell lines: Western blotting of cell lysates probed with anti-TM antiserum that recognizes multiple TMs. Positions of TM1 and TM2 are indicated. Please note that TM2 and TM3 co-migrate in this experiment. Two dimensional gel analyses of TM expression from these cell lines are published previously (7, 13–16). Normal NIH3T3 cells express all the TM isoforms, while in DT cells (and in vector control DT/V), expression of TM2 and TM3 is undetectable, with a 50% decrease in TM1 expression. In DT/TM1 cells TM1 expression is enhanced, and DT/TM2 cells overexpress TM2. DT/TM1-TM2 cells manifest elevated levels of TM1 and TM2

gelsolin antibody revealed that there is a detectable decrease (in relation to tubulin expression) in the expression in DT and DT/V cells when compared to untransformed cells. Gelsolin levels did not significantly change in DT/TM1 or DT/TM2, but are enhanced in DT/TM1-TM2 cells, to the levels found in normal fibroblasts (data not shown).

Expression of Rho-family of regulatory proteins

Ras-related Rho family of GTPases is widely recognized as the regulators of cytoskeletal integrity and gene expression (Hall, 1998; Khosravi-Far *et al*., 1998). While Rho is implicated in the formation of microfilaments, Rac regulates lamellipodia and membrane

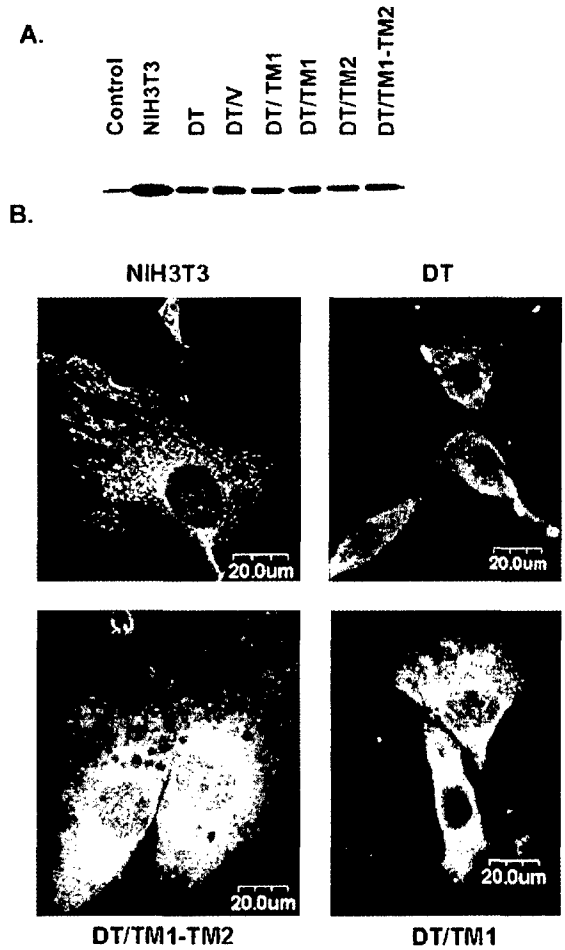

Figure 2 Vinculin expression: (**a**) Cytosolic extracts from the indicated cell lines were prepared, immunoprecipitated and Western blotted with anti-vinculin antibody, as described in Materials and methods. Control indicates a positive cell line-lysate supplied along with the antibody (Sigma chemical company). (**b**) Immunocytochemical staining of vinculin was performed with indicated cell lines. Samples were optically sectioned and the composite images were projected

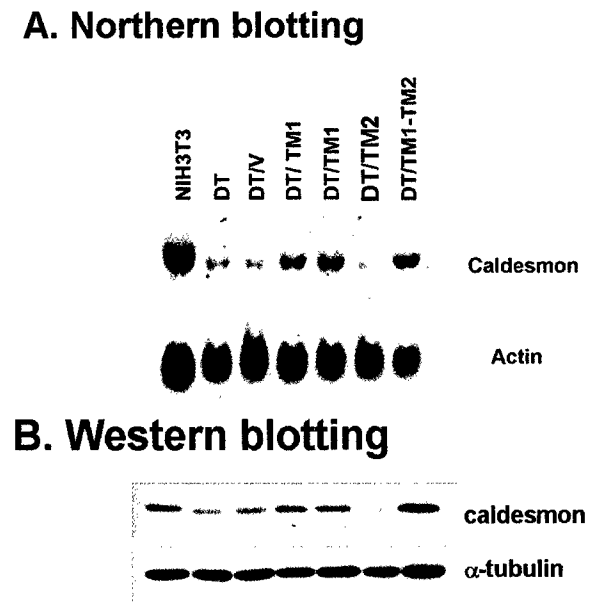

Figure 3 Caldesmon expression: (**a**) Northern blotting. 20 μg of total RNA from the indicated cell lines were loaded and hybridized against a full-length caldesmon cDNA. The membrane was stripped and reprobed with a β-actin probe for control purposes. (**b**) Western blotting. Total cytosolic extracts (100 μg) were reacted to anti-caldesmon antibody as described in Materials and methods. For control purposes, the membrane was stripped and probed with α-tubulin

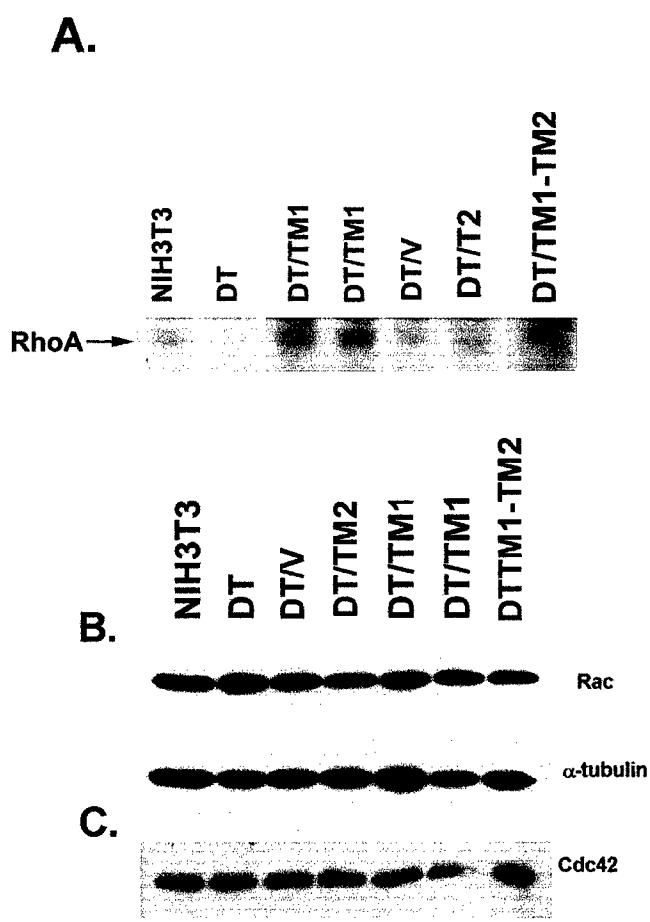

Figure 4 Expression of Rho family GTPases: (**a**) RhoA expression was determined by immunoprecipitations followed by Western blotting. Two hundred and fifty μg of total cytosolic proteins were immunoprecipitated with anti-RhoA antibodies. All the samples were immunoblotted for RhoA expression. (**b**) Rac expression was determined by immunoblotting using a monoclonal antibody (Upstate Biotechnologies) in the total cell lysates (25 μg). (**c**) Cdc42 expression was determined in 250 μg of total cytosolic proteins by immunoprecipitations and Western blotting using a monoclonal antibody (Santa Cruz Biotechnology)

ruffling and Cdc42 induces filopodia (microspikes). We investigated whether the observed reorganization and cell spreading involves changes in levels of Rho family of proteins. Immunoprecipitation followed by immunoblotting with anti-RhoA antibodies indicates that RhoA expression is lower in DT cells, while in normal fibroblasts it is readily detectable (Figure 4a). Similarly in the vector control DT cells and those expressing TM2, RhoA levels remained low. But in the DT/TM1 and DT/TM1-TM2 cells, RhoA expression is increased to normal levels. For control purposes and to test the specificity, the antibody was first reacted with the immunogen (blocking peptide) and used for immunoprecipitations (data not shown). In these experiments, as expected, no RhoA signal was detected in any of the lysates employed.

Western blotting with Rac antibody indicated a modest increase in DT cells, compared to NIH3T3 cells. But no clear-cut changes in Rac expression were discernible (Figure 4b). Immunoprecipitation and immunoblotting with anti-Cdc42 also did not reveal any appreciable changes (Figure 4c). These results indicate that TM1 expression enhances RhoA protein, which is likely to orchestrate the reorganization of cytoskeleton.

Stability of TM1 induced cytoskeleton

As noted above, the expression of many cytoskeletal proteins (including other TMs), is not completely restored in TM1-expressing DT cells. In addition, TMs are hypothesized to function as dimers; they could form homodimers as well as heterodimers. These and other interactions may not be completely restored in the revertants of DT, notwithstanding the re-emergence of microfilaments. Because of these reasons, we investigated whether TM1-induced cytoskeleton is as stable as the one in NIH3T3 cells: one established technique involves treatment of the cells with fungal toxins such as cytochalasins (Warren *et al.*, 1995). NIH3T3, DT/TM1 and DT/TM1-TM2 cells were incubated with cytochalasin D for different time periods and stained for TMs with an antibody (green) and actin (red). In normal NIH3T3 cells, after 5 min of addition of the drug, microfilaments were clearly visible and intact. After 10 min of treatment, although microfilaments were present, they appeared to be less prominent (Figure 5a). In contrast, in DT/TM1 cells, effects of cytochalasin D were profound; as early as in 5 min, the cells were rounded, with no evidence of any

organized actin filaments (Figure 5b). DT/TM1-TM2 cells were more resistant than TM1 alone expressing cells. Cytochalasin D treatment had modest, but readily detectable effects on actin microfilaments in 5 min, which were more pronounced in 10 min (Figure 5c). Accumulation of patches of actin along more defined structures is evident in 5 min of treatment, while more extensive destruction of cytoskeleton is observed in 10 min. Thus, restoration of TM1 expression is adequate to form microfilaments in DT cells, but these structures are rather labile. However, co-expression of TM2 not only significantly improves the cytoskeletal architecture, but also stabilizes microfilaments. In all of the cell lines, changes in actin staining closely mirrored that of TM. DT and DT/TM2 cells were not employed in this study, since they lack defined microfilaments to begin with.

Rho-kinase pathway

Rho-mediated contractility is critical for the assembly of stress fibers and focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). Rho kinase (p160ROCK) is a key effector of Rho-mediated signaling (Matsui *et al*., 1996; Narumiya *et al*., 1997). Among the pathways controlled by P160ROCK, regulation of microfilaments by phosphorylation of myosin light chain kinase and myosin-binding subunit of myosin phosphatase is a prominent one (Amano *et al*., 1996, 1997). Therefore, to investigate whether the re-emergence of microfilaments in TM1 expressing cells occurs through Rho kinase directed pathways, we employed two different approaches to disrupt possible p160ROCK signaling; transient transfection of DT/TM1 cells with dominant negative variants of

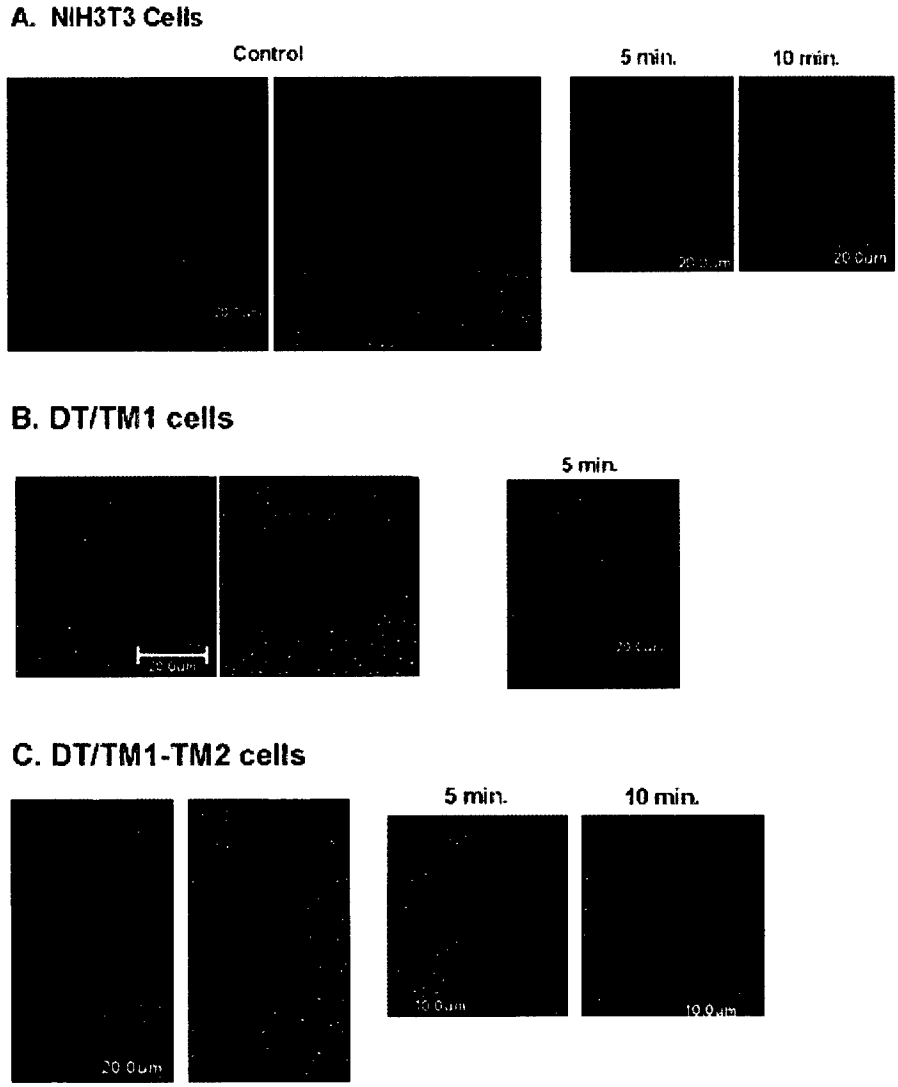

Figure 5 Stability of the cytoskeleton: NIH3T3 cells (**a**), DT/TM1 (**b**) and DT/TM1-TM2 (**c**) were treated with cytochalasin D at 0.5 μg/ml for 5 or 10 min and cells were stained for TMs and actin. Control samples were untreated. Green: TMs as visualized by anti-rabbit second antibody conjugated to FITC; and red: Actin microfilaments by Texas red conjugated phalloidin. DT/TM1 cells had essentially no cytoskeleton by 5 min of treatment and therefore, the 10 min treatment data are not shown. Since the actin-staining and TM staining was essentially identical, only actin pattern was shown for the treatments

p160Rock (Amano *et al.*, 1997), and treatment of cells with a specific inhibitor of p160ROCK, Y27632 were used (Sahai *et al.*, 1999).

We tested the importance of Rho kinase signaling in maintenance of microfilaments by transient transfection of DT/TM1-TM2 cells with a dominant negative Rho kinase construct, CAT-KD (Amano *et al.*, 1997; Chihara *et al.*, 1997). NIH3T3 cells were also transfected with CAT-KD for control purposes. In this experiment, actin filaments were visualized by staining with phalloidin: simultaneous staining for *myc* tag allowed the detection of transfected cells (Figure 6a,c), because the construct was tagged with a myc epitope. As evident from Figure 6b,d, expression of CAT-KD resulted in a severe disruption of microfilaments in NIH3T3 cells and DT/TM-TM2 cells. CAT-KD expression resulted in aggregation of actin into patches, with a profound decrease in the stress fibers. Similar results were obtained with DT/TM1 cells also (data not shown).

Treatment of NIH3T3 cells with 20 μM Y27632 for 15 min did not have significant effects on the cell spreading, but resulted in a decrease of microfilaments as observed by actin staining (Figure 7). A significant loss of microfilaments and shrinkage of cell mass was, however, evident by 30 min. Tropomyosin staining pattern was identical to that of f-actin in all the cell types also, and hence data are not presented. In DT cells, where no detectable microfilaments are absent, the effects of the drug were less obvious. Incubation with Y27632 for 15 and 30 min resulted in a similar morphology (Figure 7c,d). The spindle shaped DT cells assumed a round morphology with cytoplasmic extensions containing actin and TMs. In DT/TM1 and DT/TM1-TM2 cells, the effects of the inhibitor were far more extensive. Fifteen minutes of treatment of the drug resulted in severe disruption of microfilament network and cell morphology of the revertants (Figure 7e,g), although the effects were more pronounced in DT/TM1 cells (Figure 7e) than in DT/

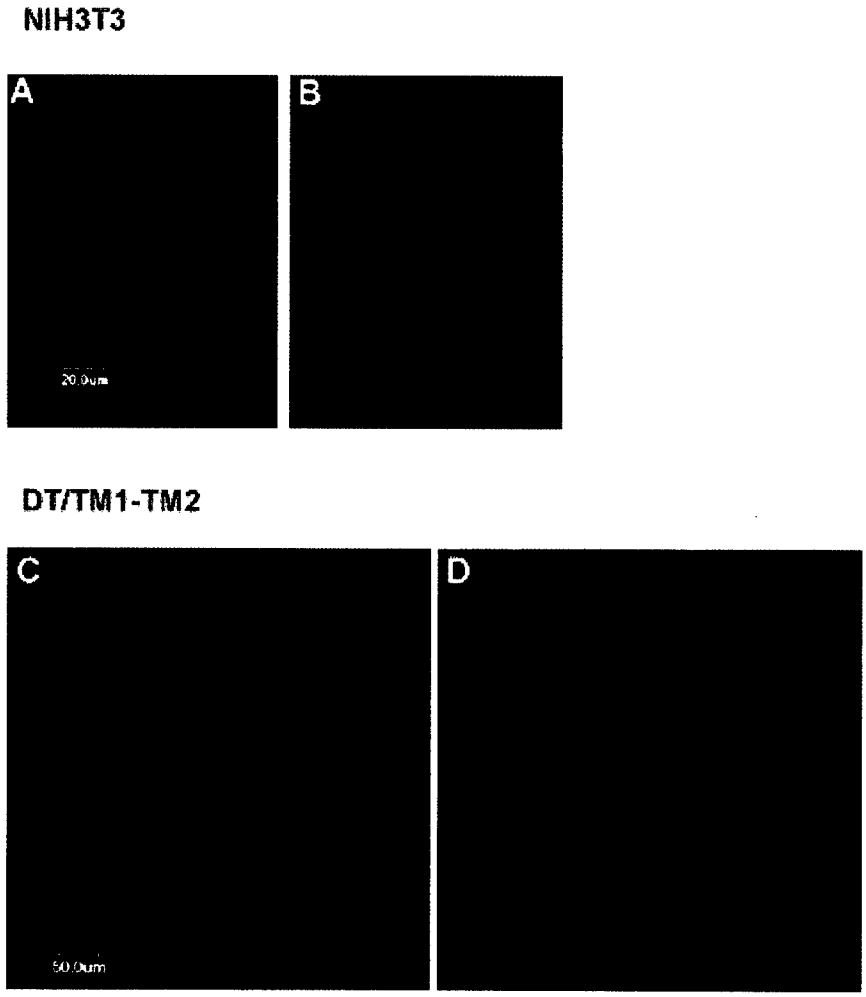

Figure 6 Effect of expression of dominant negative Rho kinase expression: Myc-epitope tagged dominant negative Rho kinase constructs (pEF-BOS-*myc* Rho kinase/CAT-KD) was used for transfection of NIH3T3 (**a,b**) and DT/TM1-TM2 (**c,d**) cells. Cells were stained for the expression of the transfected cDNA using anti-*myc*-epitope antibody (green), and microfilaments by Texas Red conjugated phalloidin. Images were viewed with an Olympus confocal microscope. Microfilament organization was severely disrupted in the transfected cells, as evidenced by patchy staining of actin, in the cells expressing CAT-KD (green)

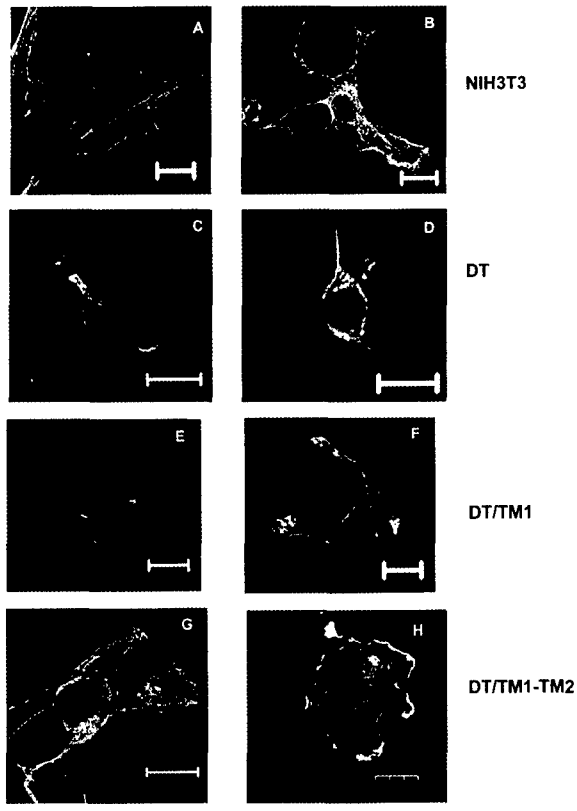

Figure 7 Effect of Y-27632 (Rho kinase inhibitor) on TM1-induced cytoskeleton: NIH3T3 (**a,b**), DT (**c,d**), DT/TM1 (**e,f**) and DT/TM1-TM2 (**g,h**) cells were treated with 20 μM of Y27632 for 15 min (**a,c,e,g**), or for 30 min (**b,d,f,h**). Cells were stained with phalloidin and photographed using a confocal microscope. The sizing bar indicates 20 μm

TM1-TM2 cells (Figure 7g). A lack of any organized microfilaments was evident in both cell types (Figure 7e–h). It should be noted that in NIH3T3 cells treated with Y-27632 a few microfilaments still remain (Figure 7b), indicating either a partial inhibition of the enzyme, or a possible participation of a different pathway. Under these conditions, however, in DT/TM1 and DT/TM1-TM2 cells complete disintegration of microfilament network is evident (Figure 7f,h). These data indicate that p160ROCK signaling is critical in the maintenance of microfilaments induced by TM1.

Discussion

Decreased expression of many actin-binding proteins, including that of TM1, in transformed cells is considered to be a contributing factor of the neoplastic phenotype (Ben-Ze'ev, 1997; Button *et al*., 1995; Janmey and Chaponnier, 1995). Restoration of TM1 expression abolishes the malignant phenotype, indicating that TM1 is a class II tumor suppressor (Prasad *et al*., 1999). Nevertheless, the underlying pathways involved in cytoskeletal reorganization by TM1 in the transformed cells are not completely understood.

We hypothesized that TM1-induced reversion of the malignant phenotype involves the restructuring of microfilaments and the modulation of signaling pathways controlled by *ras* oncogene. Reorganization of microfilaments requires participation of many other cytoskeletal proteins. In transformed cells, the levels of other actin-binding proteins, in addition to tropomyosins, are suppressed. In DT/TM1, DT/TM2 and DT/TM1-TM2 cells, expression of TM1, TM2 and both TMs is restored respectively, but the levels of other cytoskeletal proteins are not experimentally altered. In fact, expression of α-actinin, gelsolin and vinculin which are also known suppressors of transformation (Gluck *et al*., 1993; Rodriguez Fernandez *et al*., 1992) are not consistently altered in our revertant cells (data not shown). However, vinculin appeared to organize better in the revertant cells, perhaps reflecting improved microfilament architecture (Figure 2b). These data are consistent with the earlier reports that TM expression is not altered in the revertants produced by forced expression of either α-actinin or vinculin, thus suggesting the possible modes of reversion of the transformed phenotype by these cytoskeletal proteins could be distinct (Gluck *et al*., 1993; Rodriguez Fernandez *et al*., 1992).

A more notable and significant change is that caldesmon expression is specifically restored in TM1-expressing cells, but not in TM2 expressing transformed DT/TM2 cells, thus pointing to interactions between TM1 and caldesmon (Figure 3). Several lines of evidence suggest that caldesmon, which undergoes mitosis-specific phosphorylation and binds to actin in Ca^{2+}/calmodulin-regulated fashion, functions synergistically with TMs to modulate the integrity of actin cytoskeleton (Huber, 1997; Matsumura and Yamashiro, 1993); first, TMs and caldesmon together inhibit the gel severing activity of gelsolin (Ishikawa *et al*., 1989a,b); second, TMs and caldesmon enhance each others binding to actin (Ishikawa *et al*., 1998; Novy *et al*., 1993); third, caldesmon preferentially increases TM1-binding to actin when compared with that of other TM isoforms with actin (Pittenger *et al*., 1995); fourth, fascin binding with actin is shown to be completely inhibited by caldesmon and TMs together, but little or no effects were observed with either protein alone (Ishikawa *et al*., 1998); and, fifth, caldesmon when phosphorylated, or bound by calmodulin, dissociates from actin. This results in weakening of TM-actin association, exposing actin filaments to the action of gel-severing proteins (Pittenger *et al*., 1995). Furthermore, increased expression of caldesmon stabilizes microfilaments (Warren *et al*., 1995, 1996), and caldesmon levels are decreased in many transformed cells, as shown in Figure 3, and by others (Button *et al*., 1995; Novy *et al*., 1991). More recent studies implicate caldesmon in the regulation of actomyosin contractility and adhesion-dependent signaling in fibroblasts (Helfman *et al*., 1999).

TM1-induced cytoskeleton in DT cells appears to be less stable compared to that in NIH3T3 cells. The entry of several other proteins into the cytoskeletal

compartment may also remain impaired in DT/TM1 cells, due to the continued activity of *ras* oncogene in these cells. However, co-expression of TM2 resulted in a marked improvement both in the organization and stability of cytoskeleton, as judged by the sensitivity to cytochalasin D. It is known that heterodimers of TMs are thermodynamically preferred over homodimers (Jancso and Graceffa, 1991). Our previous studies showed that in DT/TM1 cells, homodimers of TM1 are formed initially (Prasad *et al.*, 1994). They are presumed to exchange with heterodimers consisting of TM1 and possibly with other low M_r TMs, since TM2 and TM3 are nearly lacking in these cells (Prasad *et al.*, 1994). In DT/TM1-TM2 cells, where both TM1 and TM2 are available, heterodimers of TM1 and TM2 are likely to exist, in addition to homodimers of TM1 and homodimers of TM2 (Shah *et al.*, 1998). These, dynamic multiple TM associations may exert more stabilizing influence and protect microfilaments.

A possible pathway through which TM1 could induce microfilaments and affect the cell phenotype is via Rho directed signaling (Narumiya *et al.*, 1997). Three different lines of evidence support the involvement of Rho proteins in the re-emergence of microfilaments: (1) RhoA expression is enhanced in TM1 expressing cells, but not in the cell lines of DT or DT/TM2 that exhibit transformed phenotype and lack microfilaments; such changes, however, were not detected in the levels of Rac1 and Cdc42; (2) inhibition of p160ROCK with specific inhibitor, Y27632, lead to the dissolution of microfilaments in normal NIH3T3 cells and TM1 expressing derivatives of DT cells; and (3) expression of a dominant negative Rho kinase, CAT-KD, interfered with the microfilament organization in NIH3T3 and DT/TM1-TM2 cells. The demonstration that RhoA is involved in TM1-induced microfilament formation complements the earlier finding that RhoA pathway may be inactivated in Ras transformation (Izawa *et al.*, 1998).

Our results are also consistent with previous data utilizing dominant active variants Rho and Rho-kinase that Rho proteins are required for the assembly of stress fibers (Izawa *et al.*, 1998). However, our results demonstrating the involvement of Rho proteins in restoration of microfilaments differ with those of other researchers who showed that Rho proteins are required for Ras transformation, and constitutively activated of RhoA could transform the cells (Khosravi-Far *et al.*, 1995; Prendergast *et al.*, 1995; Qiu *et al.*, 1995). Enhanced TM1 expression resulted in elevated RhoA proteins, and microfilaments appears to be assembled via p160ROCK pathway. Thus, in DT cells, oncogenic Ras signaling may be channeled via Rac and Cdc42 to manifest the full malignant phenotype to compensate for decreased Rho, as shown in other systems (Izawa *et al.*, 1998; Moorman *et al.*, 1999). Another possible explanation is that DT cells are very potently transformed, and the effects of oncogenic Ras signaling could be more intense. For example, DT cells form foci with $\geqslant 80\%$ efficiency in anchorage independence assays, and with inoculation of as few as 1000 cells tumor growth can be observed in 7–10 days in tumorigenesis assays (Prasad *et al.*, 1993). Notwithstanding the highly malignant nature of DT cells, restoration of TM1 expression reverts DT cells. Whether reconstituted RhoA protein is also responsible for inhibition of other downstream signaling of oncogenic Ras in this system is unclear at present, and is under investigation.

Our data indicate that p160ROCK is essential in the assembly of stress fibers in TM1-induced cytoskeletal reorganization, indicating the similarity between the pathways operating in the revertants and in normal cells. These are likely to include RhoA directed activation of Rho kinase, which in turn stimulates contractility to assemble microfilaments (Amano *et al.*, 1996; Chrzanowska-Wodnicka and Burridge, 1996). For example, Y-27632 was shown to disrupt microfilament network in NIH3T3 cells (Sahai *et al.*, 1999). In addition, transformation and cytoskeletal changes induced by constitutively active RhoA are inhibited by Y27632. The data presented herein suggest that upregulation and activation of the endogenous RhoA is likely to mediate TM1-induced cytoskeletal reorganization. The dominant negative variants of Rho kinase and Y-27632, although appear to elicit different morphological effects, are able to disrupt the stress fibers in the revertants employed in this study.

We propose that TM1-induced stress fiber assembly occurs via the following model. TM1 induces restoration of RhoA and caldesmon, reorganizes vinculin at the focal adhesions in DT cells. It is likely that the expression of caldesmon could be elevated in the revertant cells under the influence of RhoA. Caldesmon expression is shown to be regulated by SRF (Momiyama *et al.*, 1998), which in turn is regulated by RhoA (Montaner *et al.*, 1999; Spencer and Misra, 1999; Treisman *et al.*, 1998; Zohar *et al.*, 1998). Enhanced RhoA levels reactivate p160ROCK pathway, which is essential for maintenance of microfilaments. It is recently demonstrated that multiple factors, including cytoskeletal structures regulate Rho activity (Ren *et al.*, 1999). Co-expression of TM1 and TM2 significantly improves microfilament architecture and stability. Whether the restored cytoskeleton is essential in establishing and maintaining a revertant phenotype induced by TM1 is under investigation.

Materials and methods

Cell culture

Culture conditions have been described for all cell lines used in this study (Braverman *et al.*, 1996). Briefly, NIH3T3, DT cells and the derivative cell lines are cultured in DMEM high glucose medium (Life Technologies, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) in the presence of L-glutamine and penicillin and streptomycin. Cell lines derived from DT were supplemented with appropriate drugs, depending on the selection marker. The medium for DT/TM1 cells contained 200 μg/ml of Geneticin (Life Technologies), while xanthine (250 μg/ml; Sigma Chemical Company, St. Louis, MO, USA) and mycophenolic acid (2.5 mg/ml; Sigma) are present in the

culture medium for DT/TM2 and DT/TM1-TM2 cells; in addition, DT/TM1-TM2 cell media also contained G418.

Cytochalasin D (Sigma) was added to medium at (0.5 μg/ml) to test the stability of microfilaments for indicated times and the cells were processed for immunofluorescence experiments, as described below. Rho kinase inhibitor, Y-27632 was generously provided by Yoshitomi Pharmaceutical Industries, Ltd., Japan, and was added to the medium at 20 μM.

Western blotting and immunoprecipitations

Cell lysates were prepared as previously described in 1% NP-40/0.25% deoxycholate containing 50 mM Tris buffer pH 7.4 and 0.15 M NaCl, with protease inhibitors (Shah *et al*., 1998). 100 μg of protein was subjected to electrophoresis on SDS-polyacrylamide gels and electrophoretically transferred to Nytran (S and S, Keene, NH, USA). Western blotting was performed using antibodies against caldesmon, vinculin, gelsolin, α-actinin, Rac, CDC42 and RhoA proteins with appropriate second antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and Renaissance chemiluminescence kit (NEN, Boston, MA, USA). The blots were routinely reprobed upon treatment with 2% SDS and 5% 2-mercaptoethanol at 55°C with an antibody against α-tubulin for load control. Immunoprecipitations of cell lysates (250–500 μg of protein) were performed with relevant primary antibodies, and the same primary antibodies were used for detection in Western blotting.

Immunofluorescence

Cells were cultured in Nunc chamber slides, and treated with either cytochalasin D or Rho kinase inhibitor as needed. Cells were gently washed with phosphate buffered saline, fixed in 3.7% paraformaldehyde and extracted in 0.5% Triton X-100 for 15 min. The samples were incubated with the primary antibodies, followed by appropriate FITC-conjugated second antibodies (Molecular Probes, Eugene, OR, USA). When necessary the samples were also stained with phalloidin conjugated with Texas Red (Molecular Probes) and mounted in Prolong Antifade (Molecular Probes, Eugene, OR, USA) according to the manufacturer. The samples were viewed with an Olympus laser scanning confocal microscope, or a Zeiss LSM510 confocal microscope (Shah *et al*., 1998).

Northern blotting

Total RNA was extracted from cultured cells and probed with a caldesmon cDNA probe. The same blot was stripped and reprobed with βactin cDNA as described earlier to normalize for RNA loading (Prasad *et al*., 1993).

Plasmids and transfections

A dominant negative form of Rho kinase, CAT-KD was previously described (Amano *et al*., 1996; 1997; Chihara *et al*., 1997). pEF-Bos-*myc*-Rhokinase CAT-KD plasmid was used for transfection of normal NIH3T3 and DT/TM1-TM2 cells using DOTAP (Roche Molecular Biochemicals, Indianapolis, IN, USA), according to the manufacturer's guidelines. Forty eight hours after transfection, the cells were fixed and processed for immunocytochemistry as described above.

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References

Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y and Kaibuchi K. (1997). *Science*, **275**, 1308–1311.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y and Kaibuchi K. (1996). *J. Biol. Chem.*, **271**, 20246–20249.

Asch HL, Winston JS, Edge SB, Stomper PC and Asch BB. (1999). *Breast Cancer Research and Treatment*, **55**, 179–188.

Ayscough KR. (1998). *Curr. Opin. Cell Biol.*, **10**, 102–111.

Ben-Ze'ev A. (1997). *Curr. Opin. Cell Biol.*, **9**, 99–108.

Bhattacharya B, Prasad GL, Valverius EM, Salomon DS and Cooper HL. (1990). *Cancer Res.*, **50**, 2105–2112.

Braverman RH, Cooper HL, Lee HS and Prasad GL. (1996). *Oncogene*, **13**, 537–545.

Button E, Shapland C and Lawson D. (1995). *Cell Motil. Cytoskeleton*, **30**, 247–251.

Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, Ichikawa H, Ikebe R, Ikebe M and Kaibuchi K. (1997). *J. Biol. Chem.*, **272**, 25121–25127.

Chrzanowska-Wodnicka M and Burridge K. (1996). *J. Cell Biol.*, **133**, 1403–1415.

Cooper HL, Bhattacharya B, Bassin RH and Salomon DS. (1987). *Cancer Res.*, **47**, 4493–4500.

Cooper HL, Feuerstein N, Noda M and Bassin RH. (1985). *Mol. Cell Biol.*, **5**, 972–983.

Gluck U, Kwiatkowski DJ and Ben-Ze'ev A. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 383–387.

Hall A. (1998). *Science*, **279**, 509–514.

Helfman DM, Levy ET, Berthier C, Shtutman M, Riveline D, Grosheva I, Lachish-Zalait A, Elbaum M and Bershadsky AD. (1999). *Mol. Biol. Cell*, **10**, 3097–3112.

Hendricks M and Weintraub H. (1981). *Proc. Natl. Acad. Sci. USA*, **78**, 5633–5637.

Huber PA. (1997). *Int. J. Biochem. Cell Biol.*, **29**, 1047–1051.

Ishikawa R, Yamashiro S, Kohama K and Matsumura F. (1998). *J. Biol. Chem.*, **273**, 26991–26997.

Ishikawa R, Yamashiro S and Matsumura F. (1989a). *J. Biol. Chem.*, **264**, 16764–16770.

Ishikawa R, Yamashiro S and Matsumura F. (1989b). *J. Biol. Chem.*, **264**, 7490–7497.

Izawa I, Amano M, Chihara K, Yamamoto T and Kaibuchi K. (1998). *Oncogene*, **17**, 2863–2871.

Jancso A and Graceffa P. (1991). *J. Biol. Chem.*, **266,** 5891–5897.
Janmey PA and Chaponnier C. (1995). *Curr. Opin. Cell Biol.*, **7,** 111–117.
Joneson T, White MA, Wigler MH and Bar-Sagi D. (1996). *Science*, **271,** 810–812.
Khosravi-Far R, Campbell S, Rossman KL and Der CJ. (1998). *Adv. Cancer Res.*, **72,** 57–107.
Khosravi-Far R, Solski PA, Clark GJ, Kinch MS and Der CJ. (1995). *Mol. Cell. Biol.*, **15,** 6443–6453.
Kumar CC and Chang C. (1992). *Cell Growth Differ.*, **3,** 1–10.
Leavitt J, Latter G, Lutomski L, Goldstein D and Burbeck S. (1986). *Mol. Cell Biol.*, **6,** 2721–2726.
Lin JJ, Warren KS, Wamboldt DD, Wang T and Lin JL. (1997). *International Review of Cytology*, **170,** 1–38.
Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A and Kaibuchi K. (1996). *EMBO J.*, **15,** 2208–2216.
Matsumura F, Lin JJ, Yamashiro-Matsumura S, Thomas GP and Topp WC. (1983). *J. Biol. Chem.*, **258,** 13954–13964.
Matsumura F and Yamashiro S. (1993). *Curr. Opin. Cell Biol.*, **5,** 70–76.
Mielnicki LM, Ying AM, Head KL, Asch HL and Asch BB. (1999). *Exp. Cell Res.*, **249,** 161–176.
Momiyama T, Hayashi K, Obata H, Chimori Y, Nishida T, Ito T, Kamiike W, Matsuda H and Sobue K. (1998). *Biochem. Biophys. Res. Comm.*, **242,** 429–435.
Montaner S, Perona R, Saniger L and Lacal JC. (1999). *J. Biol. Chem.*, **274,** 8506–8515.
Moorman JP, Luu D, Wickham J, Bobak DA and Hahn CS. (1999). *Oncogene*, **18,** 47–57.
Narumiya S, Ishizaki T and Watanabe N. (1997). *FEBS Lett.*, **410,** 68–72.
Novy RE, Lin JL and Lin JJ. (1991). *J. Biol. Chem.*, **266,** 16917–16924.
Novy RE, Sellers JR, Liu LF and Lin JJ. (1993). *Cell Motil. Cytoskeleton*, **26,** 248–261.
Pittenger MF, Kazzaz JA and Helfman DM. (1994). *Curr. Opin. Cell Biol.*, **6,** 96–104.
Pittenger MF, Kistler A and Helfman DM. (1995). *J. Cell Sci.*, **108,** 3253–3265.
Prasad GL, Fuldner RA and Cooper HL. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 7039–7043.
Prasad GL, Fuldner RA, Braverman R, McDuffie E and Cooper HL. (1994). *Eur. J. Biol. Chem.*, **224,** 1–10.
Prasad GL, Masuelli L, Raj MH and Harindranath N. (1999). *Oncogene*, **18,** 2027–2031.
Prasad GL, Meissner PS, Sheer D and Cooper HL. (1991). *Biochem. Biophys. Res. Comm.*, **177,** 1068–1075.
Prendergast GC, Khosravi-Far R, Solski PA, Kurzawa H, Lebowitz PF and Der CJ. (1995). *Oncogene*, **10,** 2289–2296.
Qiu RG, Chen J, McCormick F and Symons M. (1995). *Proc. Natl. Acad. Sci. USA*, **92,** 11781–11785.
Ren XD, Kiosses WB and Schwartz MA. (1999). *EMBO J.*, **18,** 578–585.
Rodriguez Fernandez JL, Geiger B, Salomon D, Sabanay I, Zoller M and Ben-Ze'ev A. (1992). *J. Cell. Biol.*, **119,** 427–438.
Sahai E, Ishizaki T, Narumiya S and Treisman R. (1999). *Current Biology*, **9,** 136–145.
Shah V, Braverman R and Prasad GL. (1998). *Somatic Cell. Mol. Gen.*, **24,** 273–280.
Spencer JA and Misra RP. (1999). *Oncogene*, **18,** 7319–7327.
Treisman R, Alberts AS and Sahai E. (1998). *Cold Spring Harbor Symposia on Quantitative Biology*, **63,** 643–651.
Warren KS, Lin JL, McDermott JP and Lin JJ. (1995). *J. Cell. Biol.*, **129,** 697–708.
Warren KS, Shutt DC, McDermott JP, Lin JL, Soll DR and Lin JJ. (1996). *Cell. Motil. Cytoskeleton*, **34,** 215–229.
Zohar M, Teramoto H, Katz BZ, Yamada KM and Gutkind JS. (1998). *Oncogene*, **17,** 991–998.

TROPOMYOSIN-1 IS A SUPPRESSOR OF THE MALIGNANT PHENOTYPE OF BREAST CANCER CELLS.

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ABSTRACT

Dynamic regulation of cytoskeleton is crucial for the normal functioning of cells. Tropomyosins (TMs) are a family of cytoskeletal proteins that bind to and stabilize microfilaments. We are employing TMs as a paradigm to elucidate the role of cytoskeleton in tumorigenesis. Diverse oncogenic modalities suppress several key microfilament-associated proteins, such as TMs. Loss of expression of TMs results in the assembly of defective microfilaments leading to the abnormal morphology. These changes are hypothesized to contribute to the acquisition of the malignant growth phenotype. We identified that suppression of tropomyosin isoform 1 (TM1) as a common, yet pivotal biochemical event during the neoplastic transformation of mammary epithelial cells.

To investigate whether TM1 is a suppressor of breast cancer, MCF-7 cells are used as a model. Restoration of TM1 expression increases serum dependence, significantly decreases growth rates, but does not alter the estrogen sensitivity of the breast carcinoma cells. MCF-7 cells expressing TM1 grow as tighter colonies with a glandular morphology. TM1 expression abolishes the anchorage independent growth of MCF-7 cells, thus demonstrating that TM1 is a suppressor of the transformed phenotype of breast carcinoma cells. Enhanced TM1 expression results in relocalization of E-cadherin and β-catenin to cell-cell junctions, thus indicating a role for cadherin-catenin complexes in TM1-induced reversion of the malignant phenotype of breast cancer cells. These studies also suggest novel interactions between microfilaments and cell adhesion molecules in maintenance of normal growth phenotype of mammary epithelium.

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